A fatal attraction: macrophage recruitment to the atherosclerotic plaque

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Clinical implications

The life expectancy and disease profile of human societies are known to be correlated with the level of their economic development and social organization. Upon industrialization, the major cause of death shifted from nutritional deficiencies and infectious diseases towards degenerative diseases including cardiovascular diseases (CVD), cancer and diabetes[1]. With 1.92 million people dying each year of coronary heart disease and 1.42 million of stroke, CVD include the first and the second most common cause of death in Europe[2,3]. Both diseases are characterized by the disturbance of blood flow in coronary or cerebral arteries respectively, in the majority of the cases a result of a progressing or rupturing atherosclerotic plaque. This makes the atherosclerotic plaque an extremely important phenomenon to study.

Atherosclerosis literally means hardening of the arteries ("sclerosis") due to the accumulation of lipids, cells and connective tissue ("atheroma"). It is a progressive disease characterized by the thickening and loss of elasticity of the walls of large and medium sized arteries. Most probably almost everyone in western society suffers from this disease but its progression usually occurs without clinical symptoms because the artery compensates for the narrowing lumen by remodeling and thereby enlarging its diameter[4]. Though a chronic disease, clinical symptoms are often acute and occur either upon (partial) occlusion of an artery by a growing atherosclerotic lesion beyond the ability to compensate for it or upon rupture of an instable lesion with the formation of a thrombus. Depending on the location of the plaque, blood flow in the downstream organ will be disturbed resulting in a wide variety of symptoms. Atherosclerosis in the coronary arteries can cause angina pectoris or myocardial infarction whereas in the carotid arteries it can result in transient cerebral events or stroke. In the peripheral circulation it might cause many different manifestations including acute limb ischemia, gangrene, ischemic renal failure, hypertension, mesenteric ischemia and bowel infarction[5].

Several risk factors determine the progression of an atherosclerotic plaque, most of them well-known like a Western-type diet, smoking, hypertension, obesity, a lack of physical exercise, diabetes and age, others less known like infectious agents and elevated homocysteine levels[6-8]. Up to now, therapy strategies focus on the reduction of these risk factors, managing lipid levels with statins[9] or lowering blood pressure with ACE inhibitors and β-blockers[10]. A better knowledge of the cellular and molecular mechanisms involved in the atherogenesis and its development into a clinically relevant risk however might lead to more targeted and more efficient therapies.

Atherogenesis

Atherosclerosis is a chronic inflammatory disease[11-14] in which macrophages play a key role but also other cell types and immune mechanisms are involved[12,15-17]. It initiates when the permeability of the inner layer of the blood vessel is altered, for example by elevated levels of (modified) LDL, locally disturbed laminar blood flow or changes in shear stress. Because of this endothelial dysfunction, the vessel wall, that is only selectively permeable, suddenly becomes permeable for the LDL particles and they accumulate in the intima[18], the matrix between the endothelial and the media layer of the blood vessel, where they are trapped by an interaction with the matrix proteoglycans[19]. Another consequence of the vessel wall damage is the activation of the endothelial cells, causing them to upregulate the expression and presentation of adhesion molecules, including E-selectin, vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM). In combination with the upregulation of chemokines such as monocyte chemoattractant protein-1 (MCP-1) they stimulate monocytes to adhere to and migrate through the endothelial layer, forming a so-called fatty streak. In this subendothelial space, the monocytes proliferate and differentiate into macrophages under the influence of monocyte colony stimulating factor (M-CSF), which is also produced by the activated endothelial cells. These macrophages use their scavenger receptors, such as CD36 and scavenger receptor A (SR-A), to take up the (modified) LDL particles[20]. Macrophages that are filled with lipids, called foam cells, get activated which induces them to start producing cytokines and chemokines, creating an inflammatory milieu and attracting more macrophages and other immune cells including T cells, dendritic cells and neutrophils to the vessel wall. This influx of new cells results in a complex atherosclerotic lesion that gains in size[16,21].

As the plaque progresses, the vascular smooth muscle cells (SMC) residing in the media underneath it will start to proliferate, triggered by foam cell produced growth factors. They migrate from the media to the endothelial layer that covers the luminal side of the plaque, where they will also produce extracellular matrix constituents such as collagen and proteoglycans. This results in a thick fibrous cap covering the plaque, which reduces the risk of plaque rupture and thus is a very important aspect in the concept of plaque stability[16,21,22].

Contrary to this plaque stabilization, other processes increase the risk of plaque rupture, the most important being the death of lipid-laden macrophages and the resulting necrotic core. Foam cell apoptosis in advanced atherosclerotic plaques can be explained through combinations of several mechanisms[23,24]. Besides a lack of sufficient growth factors, there is an abundance of cytotoxic agents such as oxygen radicals, modified lipids and specific cytokines[25]. They are all able to induce caspase activation and thereby apoptosis. Another mechanism however has gained in attention. Here, excessive lipoprotein-derived cholesterol accumulation in the cells and thus also in the membrane of the endoplasmic reticulum (ER) can induce an unfolded protein response

(UPR). This ER stress can, alone[26] or in combination with a secondary signal such as pattern recognition receptor (PRR) signaling[27,28], lead to a release of Ca^{2+} from the ER lumen[26,29], a mechanism shown *in vivo* to be strongly correlated to foam cell apoptosis[30]. The remains of the apoptotic cells are cleared by the macrophages in the early stages of atherogenesis. In later stages however, the macrophages are already overfilled and unable to phagocytose these particles efficiently. The uncleared apoptotic bodies go into secondary necrosis, leading to the accumulation of cell debris and thereby the formation of a necrotic core[20]. While cell death in early lesions is considered beneficial, a growing necrotic core in more advanced plaques is associated with increased lesion instability and thus increased risk of clinical implications[31]. An additional risk comes from the tissue factor (TF) released by the necrotic cells, which can stimulate thrombus formation[32]. Other factors in the necrotic core induce neovascularization, branching microvessels from the vasa vasorum. This new microvasculature is often leaky and thereby initiator of yet another plaque destabilizing event, intraplaque hemorrhage[33].

In the end, the ratio of fibrosis over necrosis will be a strong determinant of plaque stability, so besides influencing plaque size, the shift of this ratio might be a second option for future therapeutic strategies[22].

Animal models of atherosclerosis

History

While experimental intervention possibilities in humans are limited and mostly ethically unacceptable, atherogenesis appears to be too complex for *in vitro* studies because of the many different interacting cell types in a confined, inflammatory and lipid-rich milieu. *In vitro* studies can never mimic the physiological situation accurately enough and can therefore merely be considered indicative. Therefore, the use of animal models is inevitable. During the early years, rabbits and to a lesser extend pigs and non-human primates were used to reveal the cellular background of atherogenesis[34]. Like in other biomedical research topics however, the use of mice gained in popularity[35].

Mouse models

Mice have some undeniable advantages when used in atherosclerosis and other research. First of all, mice are small, easy to handle and quick to breed. Since atherosclerosis experiments have a relatively high variability they require large groups, making the ability to house them in relatively small facilities important. Second, the mice' genetic background is well studied and many transgenic models with over-expression or deletion of specific genes exist and continue being made, which can be applied in atherosclerosis studies[36].

Mice however also have disadvantages. Because of their size, the amount of materials collected during the experiment and post-mortem, like blood and organs, are limited while the study of small tissues can be challenging. Second, however their lesions generally develop much like human lesions and contain the same cell types, some features of the human plaque still lack in mice. Since murine blood vessels are small, oxygen can diffuse more freely to all cells without the need of vasa vasorum. Therefore, their plaque hypoxia differs significantly from human lesions, also limiting its relevance in necrotic core formation and plaque neovasculogenesis[37]. Moreover, the shear stress within the vessel remains limited so reliable models for lesion rupture are not available yet[38]. Third and most important, mice don't spontaneously develop hypercholesterolemia and therefore no atherosclerosis. To overcome this, mice were initially given an atherosclerosis inducing diet containing high saturated fat, cholesterol and cholic acid salts (cholate). This cholate however can induce an inflammatory response, an enormous drawback especially when studying the role of inflammation in atherosclerosis[39]. Therefore, transgenic mice have become the model of choice.

Most transgenic atherosclerosis mouse models are developed in C57BL/6 mice because they have a relative high susceptibility for atherogenesis compared to other mouse strains[40]. The two most common models have a deletion of either the *apoe* gene encoding apolipoprotein E (ApoE) or the *ldlr* gene encoding the LDL receptor (LDLR), while other models have an overexpression of ApoB or mutant forms of ApoE, like human ApoE2[41] or ApoE*3-Leiden[42,43]. All these genetic modifications serve the purpose of raising the cholesterol levels in these mice and are most often combined with a period in which the mice are fed a diet containing high percentages of fat and cholesterol.

In an effort to speed up atherogenesis for short-term experiments or for simulation of high shear-stress, arteries can be surgically narrowed. An example is the tightening of a small silastic collar around the carotid arteries, creating a turbulent blood flow that results in downstream plaque formation[44].

In order to combine atherosclerosis proneness with other gene deficiencies, these mouse models can be crossed with mice that lack the gene of interest, creating double KO mice. When specifically studying bone marrow derived cells however, a quicker method is the bone marrow transplantation. In this case, *ldlr*$^{-/-}$ mice are irradiated in order to deplete their bone marrow cells. Subsequently, their production of new blood cells is reconstituted by injection of bone marrow from a full or cell-type restricted KO model for the gene of interest. This creates a hybrid mouse that will still develop atherosclerotic plaques but lacks the gene of interest in all or a selection of its bone marrow derived cells[45]. A different transplantation method can be used for studying changes in genetics of the vasculature during atherogenesis. Surgical transplantation of the aortic arch from a KO mouse model to an atherosclerosis prone mouse or vice versa creates hybrid mice in which the plaque and its surrounding vessel have a different genetic background from the blood and the newly infiltrating leukocytes[46,47].

Even within one mouse model, there are different locations where atherosclerosis can be quantified **(Figure 1.1)**. In one very common method, sections are cut from the aortic root. This is the location where the aorta leaves the left ventricle of the heart, immediately downstream of the tricuspid valves. Because of the very turbulent and pulsatile blood flow in this part of the aorta, atherosclerotic plaques develop relatively rapid in this location. Therefore, it is the recommended location to study when performing short-term experiments. Moreover, the growth and phenotype of these plaques is well described in literature, making it easy to evaluate effects of the experiment on these criteria[45,48]. In a similar way, sections can be cut from the aortic arch or the innominate artery, where advanced atherosclerotic plaques will appear after longer periods of high-fat diet[49]. Another common method is the *en face* atherosclerosis assessment, in which the complete aorta is isolated, stained and pinned down[50]. This provides a clear overview of plaque size and distribution throughout the aorta but should ideally be combined with aortic root analysis since it only provides basic information on the composition of the plaques, as the immunohistochemical analysis that can be performed is limited.

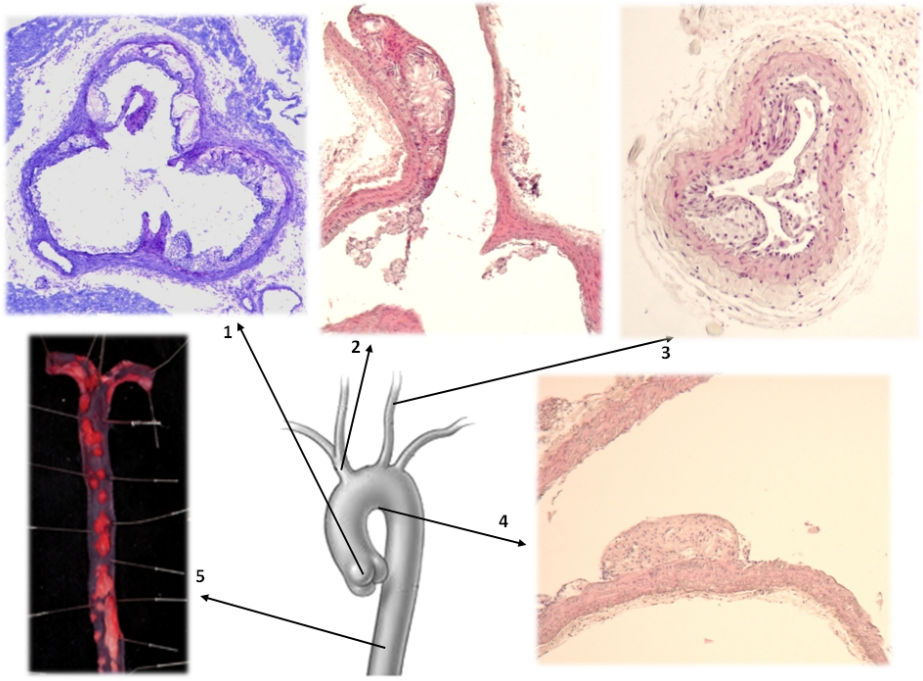

Figure 1.1*: Different locations to study atherogenesis in mouse models.* ***1****: The aortic root (Toluidine blue).* ***2****: The innominate artery (HE).* ***3****: The carotid artery, proximal of a collar (HE).* ***4****: The aortic arch (HE).* ***5****: The descending aorta* en face *(Oil Red O, image © Charles River).*

To study the gene expression within the atherosclerotic plaque, whole sections of the aorta can be lysed for RNA isolation. The results will however be disturbed by the abundant presence of RNA from the vessel wall, making the plaque-specific RNA levels in each sample dependent on the relative size of the plaque compared to the total section size. This can be solved by correcting each sample for the expression of a constant, plaque-specific gene, like the macrophage marker CD68[51]. A more precise and sensitive method however is laser capture microdissection, which makes it possible to isolate the plaques or even specific parts or cells in the plaques from histological sections, however providing a very limited RNA yield[52].

Recently, new methods emerged to study the atherosclerotic plaque composition *in vivo*, including intravital microscopy[53,54], magnetic resonance imaging (MRI)[55], fluorescence imaging (e.g. GFP or luciferase reporter systems)[56], bioluminescence imaging[57], ultrasound imaging[58] and nuclear imaging (PET and SPECT)[59]. These methods are often non- to mildly invasive and can monitor the trends of several parameters over time. Their major drawback however remains the resolution, which is at present often still inadequate to fully characterize murine plaques[60].

Macrophages

Macrophages are the most abundant and versatile cell type in atherosclerotic plaques. These immune cells with an unusual broad range of functions are formed when the bone marrow derived and in the blood circulating monocytes extravasate from the blood through the endothelial layer into the tissues, where they differentiate under the influence of macrophage- or granulocyte/macrophage colony stimulating factor (M-CSF or GM-CSF). Depending on the tissue the macrophage resides in, it may be known under a different name, with for example microglia in neural tissue, Kupffer cells in the liver, osteoclasts in the bone and sinusoidal cells in the spleen. However these macrophages usually preserve their broad range of functions independently of their location, they may as well exhibit a more specialized phenotype[61,62].

Probably the most important function of the macrophage is guarding and patrolling the tissues in search for pathogens. Once such a pathogen is detected, a series of actions follows in an attempt to clear them from the tissue. The macrophage will start to produce oxygen radicals to actively kill the pathogens and will engulf and digest the debris by phagocytosis, hence the name macrophage. At the same time, it will produce and secrete a set of cytokines and chemokines that bind to their respective receptors on the surrounding cells as a warning of the possible danger and as a trigger for the migration of other immune cells towards the site of infection. These cells include on the one hand myeloid “innate immune” cells (macrophages and granulocytes) that will assist in killing and digesting the intruders and on the other hand the antigen presenting cells (macrophages and dendritic cells) and lymphoid “adaptive immune”

cells (T- and B-cells) that will create a memory of the pathogenic patterns, which will allow the host to react more quickly and adequately upon a next encounter with the same pathogen. Upon engulfment of the pathogen, the phagosome will fuse with the lysosomes in which the pathogen is digested and antigens are formed that can be presented through MHCII molecules to the adaptive immune cells[63].

Remarkably however, the macrophage not only induces the accumulation of immune cells and an inflammatory environment, it is also responsible for the resolution of this inflammation once the pathogens are cleared. Indeed, in a second wave of cell infiltration the macrophages secrete anti-inflammatory cytokines that neutralize the inflammatory milieu and inactivate the inflammatory cells in order to protect the tissue from damage and to avoid exaggerated, systemic inflammation. Both the inability of the macrophages to raise an inflammatory response upon infection or to neutralize this response once the infection is cleared can be detrimental to the host's survival[64], as explained in chapter 5.

Similar to the phagocytosis of pathogens, also endogenous material like modified molecules, apoptotic bodies and debris of necrotic cells are cleared from the tissues by the macrophage. This way, it attempts to avoid the unnecessary inflammation that could be caused by the presence of mostly harmless agents[63].

Role of macrophages in atherosclerosis

Due to their function as scavengers, macrophages play an important role in the first phase of atherogenesis. Although patrolling already the vascular wall of healthy blood vessels, additional macrophages infiltrate the subendothelial space upon the presence of modified LDL particles[65], which they ingest using the scavenger receptors on their cell surface[66]. While this process is initially beneficial, the inability to process the particles results in lipid-laden foam cells[11]. As described above, these highly activated cells are responsible for the attraction of new macrophages and other cell types to the plaque through the production of chemokines and inflammatory cytokines but they also temper the local inflammation through the simultaneous production of anti-inflammatory cytokines. The importance of macrophages in early atherogenesis is illustrated best by experiments with mice that lack the M-CSF growth factor. These mice developed virtually no atherosclerotic plaques, as described later in this chapter[67].

In the later phases of atherogenesis, apoptosis and secondary necrosis of foam cells is causing the formation of a plaque destabilizing necrotic core while the remaining foam cells rather induce plaque stabilizing factors such as the formation of extracellular matrix and smooth muscle cell proliferation and migration[21].

Both balances between pro- and anti-inflammatory cytokine production and between plaque stability and instability are therefore highly dependent on the stimulation of the macrophage by its local environment, as further discussed in chapter 5[62]. This diversity in macrophage function however is not unique to atherosclerotic plaques but has also

been described in macrophages involved in other pathologies and physiological processes such as tumour associated macrophages (TAMs)[68], adipose tissue macrophages (ATMs)[69] and the earlier mentioned macrophages reacting to infectious diseases. It led to the concept of macrophage heterogeneity.

Macrophage heterogeneity

Recently, this macrophage heterogeneity gained a lot of attention and attempts were made to classify the different macrophage subsets into a comprehensive framework. The nomenclature makes a distinction between the pro-inflammatory, classically activated M1 and the alternatively activated, anti-inflammatory M2 phenotype, in analogy to the T helper cells which are also subdivided into Th1 and Th2 cells[70,71]. Macrophages are skewed towards an M1 phenotype by the Th1 cytokines IFNγ, TNF and/or microbial molecules such as lipopolysaccharide (LPS). They are characterized by the production of pro-inflammatory cytokines and chemokines such as IL-1β, IL-6 and TNF and anti-microbial effector molecules such as reactive oxygen and nitrogen intermediates (ROI and RNI). The M2 macrophages appeared in further study to be representing a collection of at least three different subtypes, dependent on the stimulus that induces them[72]. They are named M2a, which are induced by Th2 cytokines such as IL-4 and IL-13, M2b which are skewed by immune complexes in combination with LPS or IL-1β and M2c which are rather deactivated by cytokines such as IL-10 and transforming growth factor β (TGF-β) or by glucocorticoids.

It has shown to be difficult to find appropriate markers to identify the different macrophages, mainly because most markers described so far are not exclusively expressed but rather overexpressed in a certain subtype. Moreover, this framework is merely an oversimplified model and actual macrophages rarely display these extreme

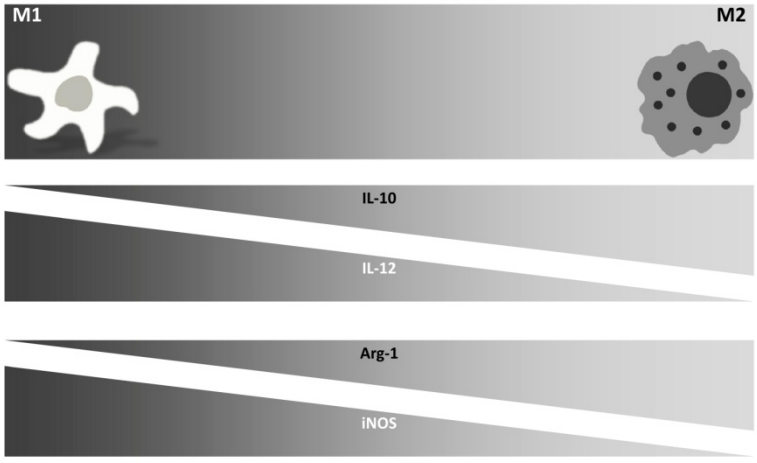

***Figure 1.2**: The IL-10/IL-12 and iNOS/arginase balances are two methods for determining the macrophage phenotype within a simplified model of heterogeneity.*

differences in phenotype. To be able to make a quick and easy distinction between M1 and M2 macrophages however, two different characterizations are described **(Figure 1.2)**. The first one is based on the inflammatory status of the macrophages and compares the expression of the pro-inflammatory cytokine IL-12, which is typically expressed in M1 macrophages, to that of the anti-inflammatory and thus M2 indicating IL-10. A second balance looks at the L-arginine metabolism and shows that this is tipped towards the expression and production of inducible nitric oxide synthase (iNOS) in M1 macrophages while the differentiation towards M2's correlates with an induction of arginase 1 (Arg-1)[73].

Different methods to induce macrophage heterogeneity in *in vitro* cell culture have been described. In the most common one, skewing cytokines (e.g. IFNγ for M1 and IL-4 for M2) are added to the culture of a macrophage cell line or macrophages obtained through the differentiation of bone marrow cells or monocytes by M-CSF. Others however differentiate bone marrow cells into distinct phenotypes by culturing them either with M-CSF or GM-CSF[74].

Other leukocytes in the plaque

While this thesis mainly focuses on macrophages, a range of other blood cell types are described to be present in the atherosclerotic plaque[75,76]. Most of them, and especially their numerous interactions, contribute to plaque progression, inflammatory phenotype and stability[14].

T lymphocytes

T lymphocytes, or simply T cells, develop through strict selection procedures from bone marrow derived thymic and peripheral hematopoietic progenitor cells and are historically subdivided into two major subtypes, primarily based on the presence of cluster of differentiation molecules CD4 and CD8 on their cell membrane. More recent studies however revealed a far more complex subdivision, including T helper cells (T_H), cytotoxic T cells (CTL), regulatory T cells (T_{reg}), natural killer T cells (NKT), memory T cells and gamma delta T cells (γδT). This extensive heterogeneity allows them, comparable to the different macrophage subsets, to possess both pro- and anti-atherogenic properties. Especially within the T_H cell subtype, the most abundant subset within the atherosclerotic lesion which can be further subdivided into T_H1 and T_H2 and other minor subtypes, this dual role is striking[77]. While T_H1 cells promote atherogenesis by the production of the cytokine IFNγ, stimulation of SMC proliferation and a stimulus for the antigen presenting cells to upregulate their major histocompatibility complex type II (MHC-II)[17], the T_H2 cells rather produce anti-atherogenic cytokines like IL-4 and IL-10 and induce the production of antibodies directed against oxLDL[78,79]. Indeed, Th1 cells

account for most of the T lymphocytes in early lesions[80] and the absence of all T cell subsets through a Rag-1 KO mainly attenuates early atherogenesis[81]. In later phases of atherogenesis however, the T_H1/ T_H2 balance can be seen as a good indicator for T cell mediated plaque progression and stability[82].

Also the other T cell subsets are shown to be present in the atherosclerotic plaque and are overall believed to promote atherosclerosis. Their specific roles in plaque progression and stability remain however somewhat elusive and demand more focused and detailed research[14,83,84].

B lymphocytes

B lymphocytes, or B cells, develop in the bone marrow and are mainly responsible for the production of antibodies. They constantly circulate the body and can therefore also be found in the vessel wall of healthy arteries. Upon atherogenesis however, they can start proliferating in the adventitia at the base of the plaque and can migrate into the plaque from the there[85-87]. Their antibody production against oxLDL associated antigens seems to coincide with a reduction in atherogenesis and T cell infiltration into the plaque[88]. Indeed, B cell deficiency through bone marrow transplantation into *ldlr*$^{-/-}$ mice promotes atherogenesis[89]. Depletion of mature B cell by a CD20-specific monoclonal antibody however results in reduced atherosclerosis, indicating that B cells can not be simply regarded as athero-protective cells[90].

Granulocytes

Granulocytes are also covering a collection of different cell types, all derived from myeloblast progenitor cells and characterized by the presence of cytoplasmic granules. While the role of eosinophils and basophils in atherogenesis is still quite unknown[75], more information on the role of neutrophils emerges. They are constantly attracted to the inflammatory regions at the luminal side of the plaque and in the adventitia lining the base of the lesion site[91]. Here they produce pro-inflammatory cytokines, which promote atherosclerosis and plaque instability, and myeloperoxidase (MPO), which might induce endothelial cell apoptosis[91,92]. Furthermore, they secrete mediators that promote the attraction of additional monocytes to the lesion[93].

At sites of plaque rupture, another type of granulocytes named mast cells can be found[94]. By degrading the athero-protective high-density lipoproteins (HDL) and impairing cholesterol efflux, these cells promote lipid accumulation and thereby plaque progression[95]. Moreover, they destabilize the plaque by apoptosis of the collagen-secreting smooth muscle cells[95] and by activation of matrix metalloproteinases (MMP)[96,97]. Furthermore, they are shown to be involved in angiotensin activation, intraplaque hemorrhage, macrophage apoptosis, vascular permeability and leukocyte recruitment to the plaques[98,99].

Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells bridging the innate with the adaptive immunity. They are formed from myeloid precursors after which they circulate the body as immature cells and finally mature upon uptake of antigens, resulting in the migration towards a lymph node and subsequent presentation of these antigens to T cells.

In atherosclerosis, vascular DC's accumulate in a GM-CSF dependent way in the intima beneath the plaque and in the shoulder regions of the plaque itself[100,101]. Here they promote plaque growth, inflammation, the attraction of T cells and their activation through antigen presentation[101,102]. Upon uptake of an antigen, they may also migrate out of the plaque towards secondary lymph nodes, where they can activate antigen specific T cells[103].

Co-localizing with the conventional DCs, a second DC phenotype can be found in the plaque shoulder regions. These plasmacytoid DCs (pDC) are not as capable in antigen endocytosis and T cell stimulation but rather react to debris from apoptotic and necrotic cells in the plaque by secreting type I interferons and stimulating $CD4^+$ T cells in an antigen independent manner to produce IFNγ and TNF-related apoptosis-inducing ligand (TRAIL), which can induce smooth muscle cell apoptosis[104-106]. Moreover, they seem able to induce T_{reg} differentiation[107].

Signal transduction

An atherosclerotic plaque is an actively changing and highly inflammatory region containing an abundance of triggers that can induce a response in the residing cells, including modified lipids, signaling molecules, oxygen radicals and debris of apoptotic and necrotic cells. The response of the cells to these changes in their environment ranges from proliferation to cell death, from lipid uptake to -efflux and from pro- to anti-inflammatory cytokine secretion, depending highly on the way the signals are transduced towards a gene expression pattern. Many different signal transduction pathways are known to be playing a role in atherogenesis and plaque stability, and their interplay offers the advantages of signal amplification, precise fine-tuning and effective feedback loops. Indeed, the availability, abundance and combination of different receptors and signaling molecules often determines the eventual response but differs between cell types or even within the heterogeneous cell types described above, explaining the multifunctionality of these cells within the plaque.

Inflammatory signaling

An interesting example of the complex signal transduction cascades that is also very relevant to this thesis is the way in which different signals within the plaque can induce

and modify an inflammatory response. In contrast to the inflammation usually seen as a reaction to pathogen-associated molecular patterns (PAMPs), atherosclerosis is considered an example of so-called sterile inflammation. Though reports of microbiological origins of atherosclerosis exist[108,109], macrophage activation within the plaque is above all triggered by autologous damage-associated molecular patterns (DAMPs), including modified lipoproteins, cholesterol crystals, cytokines and debris of necrotic or apoptotic cells[110-112].

A key mediator that regulates the expression of several pro- and anti-inflammatory genes in atherosclerosis is NF-κB[45,113,114]. This transcription factor, which in fact represents a family of different homo- or heterodimeric combinations of five different NF-κB/Rel proteins, is shown to be activated in atherosclerotic plaques, while it is usually inactive in healthy blood vessels[115,116]. In order to induce the transcription of over 160 genes, NF-κB binds to a specific consensus DNA sequence. In non-activated cells however, this interaction is impeded through tight association of an inhibitor to cytoplasmic NF-κB, preventing it to enter the nucleus. The five different proteins that make up for the inhibitor of NF-κB (IκB) family are IκBα, IκBβ, IκBγ, IκBε and Bcl-3. They are NF-κB target genes themselves, implicating a feedback loop of self-inactivation. Upon phosphorylation of the IκB inhibitors by the IκB kinase complex (IKK1, IKK2 and NEMO) followed by poly-ubiquitination and subsequent proteasomal degradation, NF-κB can freely migrate to the nucleus and influence the gene expression[117].

Within an atherosclerotic plaque, several signals can result in the activation of NF-κB and thereby evoke an inflammatory response that influences the plaque progression. The CD36 scavenger receptor can, upon oxLDL binding, heterotrimerize with Toll-like receptors (TLR) 4 and 6, initiating the same NF-κB activating pathway that can be found upon detection of viral or bacterial components by the TLR receptors[118,119]. Besides oxLDL, also the heat-shock protein HSP60 and fragments of fibronectins are TLR activating factors present in the plaque, and a similar pathway can be found downstream of the receptor for the cytokine IL-1β[120]. Indeed, deficiency of these receptors or their downstream adaptor molecule MyD88 attenuates plaque growth in murine atherosclerosis models[121-123]. Through a different signaling pathway, the binding of the inflammatory cytokine tumor necrosis factor (TNF) to its receptor (TNFR) converges with the above-mentioned pathways in the activation of the IKK complex and promotes atherosclerosis[124-127]. Also CD40, a receptor from the same family as TNFR, will bind CD40 ligand (CD40L), resulting in a similar pro-atherogenic effect[128-131]. Finally, the intracellular accumulation of cholesterol and subsequent ER stress response in foam cells was found to activate NF-κB[132], while foam cells undergoing apoptosis can induce NF-κB activation in the neighboring phagocytes[133,134].

Worth mentioning here is the strong inflammatory and pro-atherogenic cytokine IL-1β, which is produced upon NF-κB signaling in its inactive precursor form IL-1. Recently it was shown that accumulation of cholesterol crystals within the plaque macrophages stimulates the NLRP3 inflammasome complex and thereby promotes caspase-1

dependent IL-1 processing to IL-1β[135,136]. Moreover, inflammasomal caspase activation might be yet another way in which NF-κB can be activated in plaque macrophages[137].

Cytokines and chemokines

Cytokines are a family of proteins that can be produced and secreted by many different cell types upon their acute or chronic activation or are produced constitutively at low levels. The family can be divided into different classes: interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), colony stimulating factors (CSF), transforming growth factors (TGF) and chemokines[138]. Unlike hormones, they mainly act autocrine and paracrine through binding to their specific receptors, which initiates signaling pathways that lead to the activation of factors including interferon regulatory factors (IRF), janus kinase & signal transducers and activators of transcription (JAK/STAT), mitogen-activated protein (MAP) and NF-κB. While chemokines regulate attraction of new cells by creating a gradient that affects the motility and adhesion capacity of their targets and colony stimulating factors initiate maturation and proliferation of specific cell types, the other cytokines are rather messengers of the inflammatory and immune responses. Noteworthy, there are both pro- and anti-inflammatory cytokines and their balance determines the net inflammatory status of the environment. This also implicates that the different T_H- and macrophage phenotypes that have been described above can be discriminated through their profile of cytokine production.

In an atherosclerotic plaque, the co-localization of many different immune cell types combined with the presence of several stimuli like modified lipoproteins, oxygen radicals and necrotic cell remnants can induce cytokine production in the foam cells as well as in the other immune cells, the endothelial cells and the smooth muscle cells. Thereby, these cells cooperate in the creation of an inflammatory milieu that attracts and activates even more cells from the blood stream and the adventitia.

During the last 20 years, many animal experiments involving over-expression, inhibition or knock out of one of these cytokines or their receptors have proven their capital role in atherogenesis[138]. Their specific effects on atherogenesis range from altering the inflammatory and cellular balance of the lesion to plaque growth and alteration of plaque stability. These murine data can be assumed to be relevant in humans as well since similar cytokine production and cytokine receptor presence was also already shown in human atherosclerotic plaques.

Inflammatory cytokines in atherosclerosis

The inflammatory cytokine families can be sub-divided into members that mediate pro-inflammatory or anti-inflammatory effects. Their production and secretion is mainly triggered by stimuli activating the cell and influenced by a pro- or anti-inflammatory skewing of the cell. They are able to mediate both the inflammatory reaction of the

cells as well as its resolution through binding to specific receptors, initiating a range of different signaling pathways.

Because atherosclerosis is known to be a chronic inflammatory disease with many cell types mediating inflammation through the production and secretion of cytokines, their role in atherogenesis is well studied. Roughly, one could summarize that pro-inflammatory cytokines promote atherogenesis (TNF[126,139], IL-1[140-142], IL-2[143], IL-12[144,145],…) while anti-inflammatory cytokines are anti-atherogenic (IL-5[146], IL-10[147-149], IL-33[150], TGFβ[151-155],…) and others have ambiguous effects (IL-4[156-158], IL-6[159-161]). Therefore, their balance within the lesion, which is most probably dependent on the presence and activation of the different macrophage- and T helper cell subsets, is largely determining plaque growth and –stabilization. Knowledge on this balance and the specific roles of the cytokines in the plaque might reveal therapeutic possibilities, however site and/or cell type specific targeting of the therapy remains the biggest challenge.

Chemokines in atherosclerosis

Chemokines are small cytokines specialized in the chemotaxis of new cells to the site of inflammation. They share a typical three dimensional structure stabilized by disulfide bonds between two pairs of cysteines. These can be either adjacent (the CCL family) or separated by one or three other amino acids (the CXCL and CX_3CL family respectively)[162]. They play a role in the embryonic development and later in the homeostasis of tissues but their release is also often triggered by inflammatory stimuli, creating a concentration gradient able to attract specific cell subsets. Upon binding a family of G-protein coupled receptors, they induce the activation of phospholipase C (PLC). This results in the creation of the molecules inositol triphosphate (IP3) and diacylglycerol (DAG). These mediate the effects of the chemokine binding through activation of protein kinase C (PKC), the MAP kinase (MAPK) pathway and an altered intracellular Ca homeostasis. These effects include degranulation, an enhanced cell motility and a more efficient binding of the cell's integrins to adhesion molecules[162,163].

Several interactions between chemokines and their receptors influence atherogenesis[164,165], the first one discovered being the CCL2-CCR2 axis[166,167]. Later however it was shown that the attraction of new cells to the site of plaque formation mainly depends on three different and non-redundant interactions. These are CCL2 (MCP-1), CCL5 (RANTES) and CX3CL1 (Fractalkine) and their binding to the respective receptors CCR2, CCR5 and CX3CR1. Their importance was demonstrated by a simultaneous inhibition of CCL2 and the receptors CCR5 and CX3CR1, which almost completely abolished atherogenesis[168], a far more pronounced effect than the inhibition of individual chemokines or other combinations[169-172].

More recently, the focus has changed from a net effect of chemokines on atherosclerosis to a more detailed image where chemokine production differs between

early and advanced lesions and chemokine receptors are often specific to monocyte or T cell subsets. This implicates that changes in chemokine production not only affects lesion size but can also affect the attracted cell types or –subsets and thereby the plaque composition and inflammatory status[173,174].

Interferons in atherosclerosis

Interferons were the first known cytokines, discovered as the product of virus-infected cells that caused resistance to infection with the same or other viruses[175]. They are produced by lymphocytes upon contact with pathogens or tumor cells in order to alert neighbouring cells of the potential danger. Interferons are divided into two families, called type I interferons and type II interferons. They have distinct secretion profiles and functions, bind to different receptors but both signal through Jak/STAT pathways. Recent papers show a crosstalk between the two families through their effect on and use of STAT1[176].

Type I interferons include IFNα, IFNβ, IFNζ and IFNω, which all bind to a heterodimer of interferon-α receptors IFNAR1 and IFNAR2[177]. They are best known for their ability to "interfere" with viral infection. Because of this, they have been used for decades in the treatment of certain virus related diseases like Hepatitis C, often in combination with the antiviral ribavirin[178,179]. More recently however, their anti-proliferative and pro-apoptotic effects proved to be useful in the treatment of certain tumors[177,180] while their immunomodulatory properties made it the most commonly used treatment for the autoimmune disorder multiple sclerosis (MS)[181,182].

Type II interferons only include IFNγ, which binds the heterodimeric interferon-γ receptor (IFNGR1 and IFNGR2). Next to limited antiviral and antitumoral effects they mainly activate leukocytes, contributing to an inflammatory milieu.

While the pro-atherogenic role of IFNγ is well studied[183-186], type I interferons in atherosclerosis were yet poorly documented. Moreover, the few studies published failed to determine their effect on atherogenesis and clarify the mechanisms behind this[104,187,188]. Chapter 2 focuses on determining and further clarifying the effect of type I interferon signaling on mouse models for atherosclerosis and the possibility of translating these results to human atherosclerosis[51].

Colony stimulating factors in atherosclerosis

Growth factors are a family of cytokines each responsible for the differentiation and proliferation of a certain cell type. In the atherosclerotic setting, M-CSF is produced by the endothelial and smooth muscle cells and mainly ensures the differentiation of monocytes into macrophages and further macrophage proliferation[189]. Its importance in atherosclerosis was shown in mice with osteopetrotic (op) mutations. Both homozygous mice with the naturally occurring *Csfm^{op}/Csfm^{op}* (*op/op*) mutation, that

lack functional M-CSF, and mice with a heterozygous *op* mutation shown significantly reduced atherogenesis[67,190-192].

Another growth factor playing a role in atherosclerosis is GM-CSF, with both treatment and deletion approaches resulting in increased plaque size[193,194]. The exact mechanism however is yet to be revealed. Besides recruiting endothelial progenitor cells to the plaque and promoting vasa vasorum neovascularization[194-197], it regulates dendritic cell formation and proliferation in the lesions[101,198] and reduces macrophage PPARγ activity and ABCA1 expression[193].

Cell recruitment to the atherosclerotic plaque

The growth of the early atherosclerotic plaque depends largely on the attraction of new immune cells[199]. In order to enter the plaque from the blood stream, these cells need to extravasate through the endothelial cell layer, a process called diapedesis, in a way that is similar to other, non-atherosclerotic sites of inflammation[200,201]. This is typically a three-step event where the cells initially roll over the vessel wall because of a low-affinity binding to the selectins that are present on the activated endothelial cells[202,203]. Their enhanced affinity to endothelial adhesion molecules, mediated by chemokine-dependent conformational changes in their integrins, then results in firm adhesion and leukocyte arrest[204]. In a third step, the arrested leukocytes secrete proteases that degrade the extracellular matrix between the endothelial cells so they can transmigrate through the vessel wall towards the site of inflammation[205].

Interference with this diapedesis cascade, for example by blocking antibodies against adhesion molecules or integrins, has been shown to reduce the nearby inflammatory burden[206-208]. Indeed, also in atherosclerosis, a reduced leukocyte attraction results in smaller lesions[173,209-213] while an induction in recruitment promotes atherogenesis[51,214].

Quantification of new macrophages entering an atherosclerotic plaque has been challenging up to now due to a lack of good markers. In this thesis however, two novel markers are described that will facilitate the immunohistochemical detection of these cells.

Monocytes entering the atherosclerotic plaque differentiate into macrophages under the influence of the M-CSF growth factor present within the plaque. As a result, newly recruited macrophages still express monocytic markers like the one recognized by the ER-MP58 antibody[215-217]. This myeloid precursor marker has been proven useful in the detection of recently matured macrophages in several inflammatory diseases[215,218,219] and is introduced in chapter 3 as a novel marker for newly recruited macrophages in the atherosclerotic plaque.

The M-CSF dependent expression of the inhibitor of apoptosis proteins (IAP) family member survivin was already described for macrophages entering the human

atherosclerotic lesion, while surrounding cells in the plaque and blood vessel were survivin-negative[220]. In chapter 4, it is now confirmed as a novel marker for newly recruited macrophages in murine atherosclerotic plaques as well.

This thesis

The macrophage and its recruitment to the atherosclerotic plaque is a returning theme throughout this thesis. In **Chapter 2**, we show that this recruitment is enhanced in a chemokine-dependent way by type I interferons, resulting in larger lesions upon interferon treatment and smaller lesions upon defective myeloid interferon signaling. Hereby we identify the type I interferons as a novel cytokine family with a key role in atherogenesis. Also in **Chapter 3**, enhanced macrophage recruitment explains the larger plaques in mice with a myeloid deficiency of IκBα, an inhibitor of NF-κB. In this chapter we also introduce ER-MP58 as a novel marker for newly recruited macrophages in the murine atherosclerotic plaque. In **Chapter 4**, we identify another protein that is specifically expressed in macrophages entering the murine atherosclerotic plaque, survivin. Subsequently, we target it with a lentiviral based immune therapy. We show that this immunization is indeed able to reduce the lesion size, opening new possibilities for future therapies. Finally, **Chapter 5** gives an overview of the current knowledge on the different macrophage subsets and hypothesizes how this heterogeneity might affect atherogenesis.

References

1. Yusuf, S., Reddy, S., Ounpuu, S. & Anand, S. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation* **104**, 2746-2753 (2001).
2. American Heart Association international death statistics 2008. (2009).
3. Allender, S., Scarborough, P., Peto, V., Rayner, M., Leal, J., Luengo-Fernandez, R., Gray, A. European cardiovascular disease statistics 2008. in *British Heart Foundation Statistics* (ed. Foundation, B.H.) (2008).
4. Pasterkamp, G*., et al.* Paradoxical arterial wall shrinkage may contribute to luminal narrowing of human atherosclerotic femoral arteries. *Circulation* **91**, 1444-1449 (1995).
5. Hirsch, A.T*., et al.* ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation* **113**, e463-654 (2006).
6. Fruchart, J.C., Nierman, M.C., Stroes, E.S., Kastelein, J.J. & Duriez, P. New risk factors for atherosclerosis and patient risk assessment. *Circulation* **109**, III15-19 (2004).
7. Tegos, T.J., Kalodiki, E., Sabetai, M.M. & Nicolaides, A.N. The genesis of atherosclerosis and risk factors: a review. *Angiology* **52**, 89-98 (2001).
8. Assmann, G., Schulte, H. & Cullen, P. New and classical risk factors--the Munster heart study (PROCAM). *Eur J Med Res* **2**, 237-242 (1997).
9. Shepherd, J. Who should receive a statin these days? Lessons from recent clinical trials. *J Intern Med* **260**, 305-319 (2006).
10. Fogari, R. & Zoppi, A. Antihypertensive drugs and fibrinolytic function. *Am J Hypertens* **19**, 1293-1299 (2006).
11. Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868-874 (2002).
12. Ross, R. Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**, 115-126 (1999).
13. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).
14. Galkina, E. & Ley, K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* **27**, 165-197 (2009).
15. Libby, P., Ridker, P.M. & Maseri, A. Inflammation and atherosclerosis. *Circulation* **105**, 1135-1143 (2002).
16. Lusis, A.J. Atherosclerosis. *Nature* **407**, 233-241 (2000).
17. Hansson, G.K. & Libby, P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* **6**, 508-519 (2006).
18. Kinlay, S., Libby, P. & Ganz, P. Endothelial function and coronary artery disease. *Curr Opin Lipidol* **12**, 383-389 (2001).
19. Proctor, S.D. & Mamo, J.C. Intimal retention of cholesterol derived from apolipoprotein B100- and apolipoprotein B48-containing lipoproteins in carotid arteries of Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb Vasc Biol* **23**, 1595-1600 (2003).

20. Schrijvers, D.M., De Meyer, G.R., Herman, A.G. & Martinet, W. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovasc Res* **73**, 470-480 (2007).
21. Libby, P. Atherosclerosis: the new view. *Sci Am* **286**, 46-55 (2002).
22. Hennerici, M.G. The unstable plaque. *Cerebrovasc Dis* **17 Suppl 3**, 17-22 (2004).
23. Stoneman, V.E. & Bennett, M.R. Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci (Lond)* **107**, 343-354 (2004).
24. Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* **10**, 36-46 (2010).
25. Tabas, I. Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. *Cell Death Differ* **11 Suppl 1**, S12-16 (2004).
26. Li, G*., et al.* Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J Cell Biol* **186**, 783-792 (2009).
27. Devries-Seimon, T*., et al.* Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J Cell Biol* **171**, 61-73 (2005).
28. Seimon, T.A., Obstfeld, A., Moore, K.J., Golenbock, D.T. & Tabas, I. Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 19794-19799 (2006).
29. Timmins, J.M*., et al.* Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways. *The Journal of clinical investigation* **119**, 2925-2941 (2009).
30. Seimon, T. & Tabas, I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res* **50 Suppl**, S382-387 (2009).
31. Tabas, I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol* **25**, 2255-2264 (2005).
32. Taubman, M.B*., et al.* Tissue factor in the pathogenesis of atherosclerosis. *Thromb Haemost* **78**, 200-204 (1997).
33. Virmani, R*., et al.* Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol* **25**, 2054-2061 (2005).
34. Faggiotto, A. & Ross, R. Studies of hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis (Dallas, Tex* **4**, 341-356 (1984).
35. Malakoff, D. The rise of the mouse, biomedicine's model mammal. *Science* **288**, 248-253 (2000).
36. Daugherty, A. Mouse models of atherosclerosis. *The American journal of the medical sciences* **323**, 3-10 (2002).
37. Sluimer, J.C. & Daemen, M.J. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J Pathol* **218**, 7-29 (2009).
38. Cullen, P*., et al.* Rupture of the atherosclerotic plaque: does a good animal model exist? *Arterioscler Thromb Vasc Biol* **23**, 535-542 (2003).
39. Vergnes, L., Phan, J., Strauss, M., Tafuri, S. & Reue, K. Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression. *J Biol Chem* **278**, 42774-42784 (2003).

40. Paigen, B., Holmes, P.A., Mitchell, D. & Albee, D. Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H. *Atherosclerosis* **64**, 215-221 (1987).
41. Fazio, S.*, et al.* Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E(Arg 112,Cys142). *Arterioscler Thromb* **14**, 1873-1879 (1994).
42. Groot, P.H.*, et al.* Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arterioscler Thromb Vasc Biol* **16**, 926-933 (1996).
43. Leppanen, P., Luoma, J.S., Hofker, M.H., Havekes, L.M. & Yla-Herttuala, S. Characterization of atherosclerotic lesions in apo E3-leiden transgenic mice. *Atherosclerosis* **136**, 147-152 (1998).
44. von der Thusen, J.H., van Berkel, T.J. & Biessen, E.A. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation* **103**, 1164-1170 (2001).
45. Kanters, E.*, et al.* Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *The Journal of clinical investigation* **112**, 1176-1185 (2003).
46. Hu, Y. & Xu, Q. Vessel graft atherosclerosis in murine models. *Curr Drug Targets* **9**, 239-250 (2008).
47. Llodra, J.*, et al.* Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A* **101**, 11779-11784 (2004).
48. Gijbels, M.J.*, et al.* Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* **143**, 15-25 (1999).
49. Rosenfeld, M.E., Averill, M.M., Bennett, B.J. & Schwartz, S.M. Progression and disruption of advanced atherosclerotic plaques in murine models. *Curr Drug Targets* **9**, 210-216 (2008).
50. Nakashima, Y., Raines, E.W., Plump, A.S., Breslow, J.L. & Ross, R. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol* **18**, 842-851 (1998).
51. Goossens, P.*, et al.* Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. *Cell Metab* **12**, 142-153 (2010).
52. Trogan, E. & Fisher, E.A. Laser capture microdissection for analysis of macrophage gene expression from atherosclerotic lesions. *Methods Mol Biol* **293**, 221-231 (2005).
53. Zarbock, A. & Ley, K. New insights into leukocyte recruitment by intravital microscopy. *Curr Top Microbiol Immunol* **334**, 129-152 (2009).
54. Megens, R.T.*, et al.* In vivo high-resolution structural imaging of large arteries in small rodents using two-photon laser scanning microscopy. *J Biomed Opt* **15**, 011108 (2010).
55. Weinreb, D.B., Aguinaldo, J.G., Feig, J.E., Fisher, E.A. & Fayad, Z.A. Non-invasive MRI of mouse models of atherosclerosis. *NMR Biomed* **20**, 256-264 (2007).
56. Partridge, J.*, et al.* Laminar shear stress acts as a switch to regulate divergent functions of NF-kappaB in endothelial cells. *FASEB J* **21**, 3553-3561 (2007).
57. Gross, S.*, et al.* Bioluminescence imaging of myeloperoxidase activity in vivo. *Nature medicine* **15**, 455-461 (2009).
58. Kornmann, L.M.*, et al.* Perfluorohexane-loaded macrophages as a novel ultrasound contrast agent: a feasibility study. *Mol Imaging Biol* **10**, 264-270 (2008).
59. Breyholz, H.J.*, et al.* Radiofluorinated pyrimidine-2,4,6-triones as molecular probes for noninvasive MMP-targeted imaging. *ChemMedChem* **5**, 777-789 (2010).

60. Jaffer, F.A. & Weissleder, R. Seeing within: molecular imaging of the cardiovascular system. *Circ Res* **94**, 433-445 (2004).
61. Geissmann, F*., et al.* Development of monocytes, macrophages, and dendritic cells. *Science* **327**, 656-661 (2010).
62. Stoger, J.L., Goossens, P. & de Winther, M.P. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. *Curr Vasc Pharmacol* **8**, 233-248 (2010).
63. Gordon, S. The macrophage: past, present and future. *Eur J Immunol* **37 Suppl 1**, S9-17 (2007).
64. Namangala, B., Noel, W., De Baetselier, P., Brys, L. & Beschin, A. Relative contribution of interferon-gamma and interleukin-10 to resistance to murine African trypanosomosis. *J Infect Dis* **183**, 1794-1800 (2001).
65. Galkina, E*., et al.* Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med* **203**, 1273-1282 (2006).
66. Peiser, L., Mukhopadhyay, S. & Gordon, S. Scavenger receptors in innate immunity. *Curr Opin Immunol* **14**, 123-128 (2002).
67. Smith, J.D*., et al.* Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8264-8268 (1995).
68. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **23**, 549-555 (2002).
69. Harman-Boehm, I*., et al.* Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab* **92**, 2240-2247 (2007).
70. Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J. & Hill, A.M. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* **164**, 6166-6173 (2000).
71. Mantovani, A., Sica, A. & Locati, M. Macrophage polarization comes of age. *Immunity* **23**, 344-346 (2005).
72. Mantovani, A*., et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **25**, 677-686 (2004).
73. Hesse, M*., et al.* Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* **167**, 6533-6544 (2001).
74. Fleetwood, A.J., Dinh, H., Cook, A.D., Hertzog, P.J. & Hamilton, J.A. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J Leukoc Biol* **86**, 411-421 (2009).
75. Weber, C., Zernecke, A. & Libby, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* **8**, 802-815 (2008).
76. Vanderlaan, P.A. & Reardon, C.A. Thematic review series: the immune system and atherogenesis. The unusual suspects:an overview of the minor leukocyte populations in atherosclerosis. *J Lipid Res* **46**, 829-838 (2005).
77. Frostegard, J*., et al.* Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* **145**, 33-43 (1999).
78. Buono, C*., et al.* T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc Natl Acad Sci U S A* **102**, 1596-1601 (2005).
79. Robertson, A.K. & Hansson, G.K. T cells in atherogenesis: for better or for worse? *Arterioscler Thromb Vasc Biol* **26**, 2421-2432 (2006).

80. Zhou, X., Paulsson, G., Stemme, S. & Hansson, G.K. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *The Journal of clinical investigation* **101**, 1717-1725 (1998).
81. Song, L., Leung, C. & Schindler, C. Lymphocytes are important in early atherosclerosis. *J Clin Invest* **108**, 251-259 (2001).
82. Taleb, S., Tedgui, A. & Mallat, Z. Adaptive T cell immune responses and atherogenesis. *Curr Opin Pharmacol* **10**, 197-202 (2010).
83. Hansson, G.K. Inflammatory mechanisms in atherosclerosis. *J Thromb Haemost* **7 Suppl 1**, 328-331 (2009).
84. Taleb, S., Tedgui, A. & Mallat, Z. Regulatory T-cell immunity and its relevance to atherosclerosis. *J Intern Med* **263**, 489-499 (2008).
85. Moos, M.P.*, et al.* The lamina adventitia is the major site of immune cell accumulation in standard chow-fed apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **25**, 2386-2391 (2005).
86. Zhou, X. & Hansson, G.K. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. *Scand J Immunol* **50**, 25-30 (1999).
87. Watanabe, M.*, et al.* Distribution of inflammatory cells in adventitia changed with advancing atherosclerosis of human coronary artery. *J Atheroscler Thromb* **14**, 325-331 (2007).
88. Caligiuri, G., Nicoletti, A., Poirier, B. & Hansson, G.K. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *The Journal of clinical investigation* **109**, 745-753 (2002).
89. Major, A.S., Fazio, S. & Linton, M.F. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler Thromb Vasc Biol* **22**, 1892-1898 (2002).
90. Ait-Oufella, H.*, et al.* B cell depletion reduces the development of atherosclerosis in mice. *J Exp Med* **207**, 1579-1587 (2010).
91. Zernecke, A.*, et al.* Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circ Res* **102**, 209-217 (2008).
92. van Leeuwen, M.*, et al.* Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. *Arterioscler Thromb Vasc Biol* **28**, 84-89 (2008).
93. Soehnlein, O.*, et al.* Neutrophil secretion products pave the way for inflammatory monocytes. *Blood* **112**, 1461-1471 (2008).
94. Jeziorska, M., McCollum, C. & Woolley, D.E. Mast cell distribution, activation, and phenotype in atherosclerotic lesions of human carotid arteries. *J Pathol* **182**, 115-122 (1997).
95. Kovanen, P.T. Mast cells: multipotent local effector cells in atherothrombosis. *Immunol Rev* **217**, 105-122 (2007).
96. Johnson, J.L., Jackson, C.L., Angelini, G.D. & George, S.J. Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* **18**, 1707-1715 (1998).
97. Newby, A.C. & Johnson, J.L. Genetic strategies to elucidate the roles of matrix metalloproteinases in atherosclerotic plaque growth and stability. *Circ Res* **97**, 958-960 (2005).
98. Bot, I.*, et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115**, 2516-2525 (2007).
99. Caughey, G.H. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev* **217**, 141-154 (2007).
100. Liu, P.*, et al.* CX3CR1 deficiency impairs dendritic cell accumulation in arterial intima and reduces atherosclerotic burden. *Arterioscler Thromb Vasc Biol* **28**, 243-250 (2008).

101. Shaposhnik, Z., Wang, X., Weinstein, M., Bennett, B.J. & Lusis, A.J. Granulocyte macrophage colony-stimulating factor regulates dendritic cell content of atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* **27**, 621-627 (2007).
102. Zernecke, A., Shagdarsuren, E. & Weber, C. Chemokines in atherosclerosis: an update. *Arterioscler Thromb Vasc Biol* **28**, 1897-1908 (2008).
103. Randolph, G.J., Jakubzick, C. & Qu, C. Antigen presentation by monocytes and monocyte-derived cells. *Curr Opin Immunol* **20**, 52-60 (2008).
104. Niessner, A.*, et al.* Synergistic proinflammatory effects of the antiviral cytokine interferon-alpha and Toll-like receptor 4 ligands in the atherosclerotic plaque. *Circulation* **116**, 2043-2052 (2007).
105. Niessner, A.*, et al.* Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. *Circulation* **114**, 2482-2489 (2006).
106. Colonna, M., Trinchieri, G. & Liu, Y.J. Plasmacytoid dendritic cells in immunity. *Nat Immunol* **5**, 1219-1226 (2004).
107. Ochando, J.C.*, et al.* Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* **7**, 652-662 (2006).
108. Hoymans, V.Y., Bosmans, J.M., Ieven, M.M. & Vrints, C.J. Chlamydia pneumoniae-based atherosclerosis: a smoking gun. *Acta Cardiol* **62**, 565-571 (2007).
109. Erridge, C. The roles of pathogen-associated molecular patterns in atherosclerosis. *Trends Cardiovasc Med* **18**, 52-56 (2008).
110. Stepankova, R.*, et al.* Absence of microbiota (germ-free conditions) accelerates the atherosclerosis in ApoE-deficient mice fed standard low cholesterol diet. *J Atheroscler Thromb* **17**, 796-804 (2010).
111. Erridge, C. The roles of Toll-like receptors in atherosclerosis. *J Innate Immun* **1**, 340-349 (2009).
112. Wright, S.D.*, et al.* Infectious agents are not necessary for murine atherogenesis. *J Exp Med* **191**, 1437-1442 (2000).
113. Xanthoulea, S., Curfs, D.M., Hofker, M.H. & de Winther, M.P. Nuclear factor kappa B signaling in macrophage function and atherogenesis. *Curr Opin Lipidol* **16**, 536-542 (2005).
114. de Winther, M.P., Kanters, E., Kraal, G. & Hofker, M.H. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* **25**, 904-914 (2005).
115. Brand, K.*, et al.* Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest* **97**, 1715-1722 (1996).
116. Hajra, L.*, et al.* The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci U S A* **97**, 9052-9057 (2000).
117. Bonizzi, G. & Karin, M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**, 280-288 (2004).
118. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**, 373-384 (2010).
119. Stewart, C.R.*, et al.* CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol* **11**, 155-161 (2010).
120. Michelsen, K.S., Doherty, T.M., Shah, P.K. & Arditi, M. TLR signaling: an emerging bridge from innate immunity to atherogenesis. *J Immunol* **173**, 5901-5907 (2004).
121. Chi, H., Messas, E., Levine, R.A., Graves, D.T. & Amar, S. Interleukin-1 receptor signaling mediates atherosclerosis associated with bacterial exposure and/or a high-fat diet in a murine apolipoprotein E heterozygote model: pharmacotherapeutic implications. *Circulation* **110**, 1678-1685 (2004).

122. Michelsen, K.S*., et al.* Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci U S A* **101**, 10679-10684 (2004).
123. Bjorkbacka, H*., et al.* Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. *Nat Med* **10**, 416-421 (2004).
124. Xanthoulea, S*., et al.* Absence of p55 TNF receptor reduces atherosclerosis, but has no major effect on angiotensin II induced aneurysms in LDL receptor deficient mice. *PLoS One* **4**, e6113 (2009).
125. Xanthoulea, S*., et al.* P55 tumour necrosis factor receptor in bone marrow-derived cells promotes atherosclerosis development in low-density lipoprotein receptor knock-out mice. *Cardiovasc Res* **80**, 309-318 (2008).
126. Ohta, H*., et al.* Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis* **180**, 11-17 (2005).
127. Boesten, L.S*., et al.* Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. *Cardiovasc Res* **66**, 179-185 (2005).
128. Monaco, C*., et al.* Canonical pathway of nuclear factor kappa B activation selectively regulates proinflammatory and prothrombotic responses in human atherosclerosis. *Proc Natl Acad Sci U S A* **101**, 5634-5639 (2004).
129. Lievens, D*., et al.* Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. *Blood* **116**, 4317-4327 (2010).
130. Lutgens, E*., et al.* Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by skewing the immune response toward an antiinflammatory profile. *J Exp Med* **207**, 391-404 (2010).
131. Lievens, D., Eijgelaar, W.J., Biessen, E.A., Daemen, M.J. & Lutgens, E. The multi-functionality of CD40L and its receptor CD40 in atherosclerosis. *Thromb Haemost* **102**, 206-214 (2009).
132. Li, Y*., et al.* Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis. *J Biol Chem* **280**, 21763-21772 (2005).
133. Li, Y*., et al.* Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the Mer receptor. *J Biol Chem* **281**, 6707-6717 (2006).
134. Kono, H., Karmarkar, D., Iwakura, Y. & Rock, K.L. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. *J Immunol* **184**, 4470-4478 (2010).
135. Duewell, P*., et al.* NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* **464**, 1357-1361 (2010).
136. Rajamaki, K*., et al.* Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS One* **5**, e11765 (2010).
137. Lamkanfi, M., Declercq, W., Vanden Berghe, T. & Vandenabeele, P. Caspases leave the beaten track: caspase-mediated activation of NF-kappaB. *J Cell Biol* **173**, 165-171 (2006).
138. Tedgui, A. & Mallat, Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* **86**, 515-581 (2006).
139. Canault, M*., et al.* Progression of atherosclerosis in ApoE-deficient mice that express distinct molecular forms of TNF-alpha. *J Pathol* **214**, 574-583 (2008).

140. Devlin, C.M., Kuriakose, G., Hirsch, E. & Tabas, I. Genetic alterations of IL-1 receptor antagonist in mice affect plasma cholesterol level and foam cell lesion size. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 6280-6285 (2002).
141. Merhi-Soussi, F.*, et al.* Interleukin-1 plays a major role in vascular inflammation and atherosclerosis in male apolipoprotein E-knockout mice. *Cardiovasc Res* **66**, 583-593 (2005).
142. Kirii, H.*, et al.* Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* **23**, 656-660 (2003).
143. Upadhya, S., Mooteri, S., Peckham, N. & Pai, R.G. Atherogenic effect of interleukin-2 and antiatherogenic effect of interleukin-2 antibody in apo-E-deficient mice. *Angiology* **55**, 289-294 (2004).
144. Lee, T.S., Yen, H.C., Pan, C.C. & Chau, L.Y. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* **19**, 734-742 (1999).
145. Zhang, X.*, et al.* Interleukin 12 induces T-cell recruitment into the atherosclerotic plaque. *Circ Res* **98**, 524-531 (2006).
146. Binder, C.J.*, et al.* The role of natural antibodies in atherogenesis. *J Lipid Res* **46**, 1353-1363 (2005).
147. Caligiuri, G.*, et al.* Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med* **9**, 10-17 (2003).
148. Potteaux, S.*, et al.* Leukocyte-derived interleukin 10 is required for protection against atherosclerosis in low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* **24**, 1474-1478 (2004).
149. Von Der Thusen, J.H.*, et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J* **15**, 2730-2732 (2001).
150. Miller, A.M.*, et al.* IL-33 reduces the development of atherosclerosis. *J Exp Med* **205**, 339-346 (2008).
151. Robertson, A.K.*, et al.* Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *The Journal of clinical investigation* **112**, 1342-1350 (2003).
152. Gojova, A.*, et al.* Specific abrogation of transforming growth factor-beta signaling in T cells alters atherosclerotic lesion size and composition in mice. *Blood* **102**, 4052-4058 (2003).
153. Lutgens, E. & Daemen, M.J. Transforming growth factor-beta: a local or systemic mediator of plaque stability? *Circ Res* **89**, 853-855 (2001).
154. Lutgens, E.*, et al.* Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. *Arterioscler Thromb Vasc Biol* **22**, 975-982 (2002).
155. Mallat, Z.*, et al.* Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res* **89**, 930-934 (2001).
156. Davenport, P. & Tipping, P.G. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Pathol* **163**, 1117-1125 (2003).
157. George, J.*, et al.* Interleukin (IL)-4 deficiency does not influence fatty streak formation in C57BL/6 mice. *Atherosclerosis* **153**, 403-411 (2000).
158. George, J.*, et al.* Requisite role for interleukin-4 in the acceleration of fatty streaks induced by heat shock protein 65 or Mycobacterium tuberculosis. *Circ Res* **86**, 1203-1210 (2000).

159. Elhage, R.*, et al.* Involvement of interleukin-6 in atherosclerosis but not in the prevention of fatty streak formation by 17beta-estradiol in apolipoprotein E-deficient mice. *Atherosclerosis* **156**, 315-320 (2001).
160. Huber, S.A., Sakkinen, P., Conze, D., Hardin, N. & Tracy, R. Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* **19**, 2364-2367 (1999).
161. Schieffer, B.*, et al.* Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. *Circulation* **110**, 3493-3500 (2004).
162. Charo, I.F. & Ransohoff, R.M. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* **354**, 610-621 (2006).
163. Murdoch, C. & Finn, A. Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95**, 3032-3043 (2000).
164. Terkeltaub, R., Boisvert, W.A. & Curtiss, L.K. Chemokines and atherosclerosis. *Curr Opin Lipidol* **9**, 397-405 (1998).
165. Lucas, A.D. & Greaves, D.R. Atherosclerosis: role of chemokines and macrophages. *Expert Rev Mol Med* **3**, 1-18 (2001).
166. Boring, L., Gosling, J., Cleary, M. & Charo, I.F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**, 894-897 (1998).
167. Gu, L.*, et al.* Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* **2**, 275-281 (1998).
168. Combadiere, C.*, et al.* Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation* **117**, 1649-1657 (2008).
169. Saederup, N., Chan, L., Lira, S.A. & Charo, I.F. Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2-/- mice: evidence for independent chemokine functions in atherogenesis. *Circulation* **117**, 1642-1648 (2008).
170. Veillard, N.R.*, et al.* Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* **94**, 253-261 (2004).
171. Zhao, Q. Dual targeting of CCR2 and CCR5: therapeutic potential for immunologic and cardiovascular diseases. *J Leukoc Biol* ***88****(1), 41-55 (2010)*.
172. Potteaux, S.*, et al.* Role of bone marrow-derived CC-chemokine receptor 5 in the development of atherosclerosis of low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* **26**, 1858-1863 (2006).
173. Koenen, R.R. & Weber, C. Therapeutic targeting of chemokine interactions in atherosclerosis. *Nat Rev Drug Discov* **9**, 141-153 (2010).
174. Gautier, E.L., Jakubzick, C. & Randolph, G.J. Regulation of the migration and survival of monocyte subsets by chemokine receptors and its relevance to atherosclerosis. *Arterioscler Thromb Vasc Biol* **29**, 1412-1418 (2009).
175. Isaacs, A. & Lindenmann, J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* **147**, 258-267 (1957).
176. Gough, D.J.*, et al.* Functional crosstalk between type I and II interferon through the regulated expression of STAT1. *PLoS Biol* **8**, e1000361 (2010).
177. Borden, E.C.*, et al.* Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* **6**, 975-990 (2007).
178. Foster, G.R. Past, present, and future hepatitis C treatments. *Semin Liver Dis* **24 Suppl 2**, 97-104 (2004).
179. Heathcote, J. & Main, J. Treatment of hepatitis C. *J Viral Hepat* **12**, 223-235 (2005).
180. Chawla-Sarkar, M.*, et al.* Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* **8**, 237-249 (2003).

181. Weinstock-Guttman, B., Ramanathan, M. & Zivadinov, R. Interferon-beta treatment for relapsing multiple sclerosis. *Expert Opin Biol Ther* **8**, 1435-1447 (2008).
182. Bermel, R.A. & Rudick, R.A. Interferon-beta treatment for multiple sclerosis. *Neurotherapeutics* **4**, 633-646 (2007).
183. Gupta, S.*, et al.* IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *The Journal of clinical investigation* **99**, 2752-2761 (1997).
184. Whitman, S.C., Ravisankar, P., Elam, H. & Daugherty, A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. *Am J Pathol* **157**, 1819-1824 (2000).
185. Buono, C.*, et al.* Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse. *Arterioscler Thromb Vasc Biol* **23**, 454-460 (2003).
186. Koga, M.*, et al.* Inhibition of progression and stabilization of plaques by postnatal interferon-gamma function blocking in ApoE-knockout mice. *Circ Res* **101**, 348-356 (2007).
187. Levy, Z.*, et al.* Low-dose interferon-alpha accelerates atherosclerosis in an LDL receptor-deficient mouse model. *European journal of internal medicine* **14**, 479-483 (2003).
188. Zhang, L.N.*, et al.* Interferon-beta attenuates angiotensin II-accelerated atherosclerosis and vascular remodeling in apolipoprotein E deficient mice. *Atherosclerosis* **197**, 204-211 (2008).
189. Rosenfeld, M.E. & Ross, R. Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis (Dallas, Tex* **10**, 680-687 (1990).
190. Qiao, J.H.*, et al.* Role of macrophage colony-stimulating factor in atherosclerosis: studies of osteopetrotic mice. *Am J Pathol* **150**, 1687-1699 (1997).
191. de Villiers, W.J.*, et al.* Macrophage phenotype in mice deficient in both macrophage-colony-stimulating factor (op) and apolipoprotein E. *Arterioscler Thromb Vasc Biol* **18**, 631-640 (1998).
192. Rajavashisth, T.*, et al.* Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor- deficient mice. *The Journal of clinical investigation* **101**, 2702-2710 (1998).
193. Ditiatkovski, M., Toh, B.H. & Bobik, A. GM-CSF deficiency reduces macrophage PPAR-gamma expression and aggravates atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* **26**, 2337-2344 (2006).
194. Haghighat, A., Weiss, D., Whalin, M.K., Cowan, D.P. & Taylor, W.R. Granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor exacerbate atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **115**, 2049-2054 (2007).
195. Takahashi, T.*, et al.* Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nature medicine* **5**, 434-438 (1999).
196. Urbich, C. & Dimmeler, S. Endothelial progenitor cells: characterization and role in vascular biology. *Circulation research* **95**, 343-353 (2004).
197. Hristov, M. & Weber, C. Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *Journal of cellular and molecular medicine* **8**, 498-508 (2004).
198. Zhu, S.N., Chen, M., Jongstra-Bilen, J. & Cybulsky, M.I. GM-CSF regulates intimal cell proliferation in nascent atherosclerotic lesions. *J Exp Med* **206**, 2141-2149 (2009).
199. Mach, F. The role of chemokines in atherosclerosis. *Curr Atheroscler Rep* **3**, 243-251 (2001).

200. Cybulsky, M.I., Won, D. & Haidari, M. Leukocyte recruitment to atherosclerotic lesions. *Can J Cardiol* **20 Suppl B**, 24B-28B (2004).
201. Weber, C. Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules. *J Mol Med* **81**, 4-19 (2003).
202. Ley, K.*, et al.* Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J Exp Med* **181**, 669-675 (1995).
203. Ramos, C.L.*, et al.* Direct demonstration of P-selectin- and VCAM-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein E-deficient mice. *Circ Res* **84**, 1237-1244 (1999).
204. Hyduk, S.J. & Cybulsky, M.I. Role of alpha4beta1 integrins in chemokine-induced monocyte arrest under conditions of shear stress. *Microcirculation* **16**, 17-30 (2009).
205. Ley, K. Molecular mechanisms of leukocyte recruitment in the inflammatory process. *Cardiovasc Res* **32**, 733-742 (1996).
206. Pizcueta, P. & Luscinskas, F.W. Monoclonal antibody blockade of L-selectin inhibits mononuclear leukocyte recruitment to inflammatory sites in vivo. *Am J Pathol* **145**, 461-469 (1994).
207. Rychly, J. & Nebe, B. Therapeutic strategies in autoimmune diseases by interfering with leukocyte endothelium interaction. *Curr Pharm Des* **12**, 3799-3806 (2006).
208. Gonzalez-Amaro, R., Diaz-Gonzalez, F. & Sanchez-Madrid, F. Adhesion molecules in inflammatory diseases. *Drugs* **56**, 977-988 (1998).
209. Nageh, M.F.*, et al.* Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* **17**, 1517-1520 (1997).
210. Johnson, R.C.*, et al.* Absence of P-selectin delays fatty streak formation in mice. *J Clin Invest* **99**, 1037-1043 (1997).
211. Collins, R.G.*, et al.* P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med* **191**, 189-194 (2000).
212. Dong, Z.M.*, et al.* The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest* **102**, 145-152 (1998).
213. Haverslag, R., Pasterkamp, G. & Hoefer, I.E. Targeting adhesion molecules in cardiovascular disorders. *Cardiovasc Hematol Disord Drug Targets* **8**, 252-260 (2008).
214. von Hundelshausen, P., Koenen, R.R. & Weber, C. Platelet-mediated enhancement of leukocyte adhesion. *Microcirculation* **16**, 84-96 (2009).
215. Chan, J., Leenen, P.J., Bertoncello, I., Nishikawa, S.I. & Hamilton, J.A. Macrophage lineage cells in inflammation: characterization by colony-stimulating factor-1 (CSF-1) receptor (c-Fms), ER-MP58, and ER-MP20 (Ly-6C) expression. *Blood* **92**, 1423-1431 (1998).
216. Leenen, P.J., de Bruijn, M.F., Voerman, J.S., Campbell, P.A. & van Ewijk, W. Markers of mouse macrophage development detected by monoclonal antibodies. *J Immunol Methods* **174**, 5-19 (1994).
217. Sunderkotter, C.*, et al.* Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* **172**, 4410-4417 (2004).
218. Cook, A.D., Braine, E.L. & Hamilton, J.A. The phenotype of inflammatory macrophages is stimulus dependent: implications for the nature of the inflammatory response. *J Immunol* **171**, 4816-4823 (2003).
219. Yamamoto, T.*, et al.* Repopulation of murine Kupffer cells after intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. *Am J Pathol* **149**, 1271-1286 (1996).

220. Blanc-Brude, O.P.*, et al.* IAP survivin regulates atherosclerotic macrophage survival. *Arterioscler Thromb Vasc Biol* **27**, 901-907 (2007).

CHAPTER 2

Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions

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Abstract

Inflammatory cytokines are well-recognized mediators of atherosclerosis. Depending on the pathological context, type I interferons (IFNs; IFNα and IFNβ) exert either pro- or anti-inflammatory immune functions but their exact role in atherogenesis has not been clarified. Here we demonstrate that IFNβ enhances macrophage-endothelial cell adhesion and promotes leukocyte attraction to atherosclerosis prone sites in mice in a chemokine-dependent manner. Moreover, IFNβ treatment accelerates lesion formation in two different mouse models of atherosclerosis and increases macrophage accumulation in the plaques. Concomitantly, absence of endogenous type I IFN signaling in myeloid cells inhibits lesion development, protects against lesional accumulation of macrophages and prevents necrotic core formation. Finally, we show that type I IFN signaling is upregulated in ruptured human atherosclerotic plaques. Hereby, we identify type I IFNs as novel pro-atherosclerotic cytokines, which may serve as additional targets for prevention or treatment.

Introduction

The important contribution of inflammatory cytokines to atherosclerosis development is well recognized[1-3]. The macrophage is both an important source as well as a major target of these inflammatory mediators. Specific cytokines influence macrophage effector functions and thereby affect plaque initiation, progression and cellular composition. Ultimately, macrophages hereby alter important plaque stability factors such as necrotic core formation and inflammatory cell content. Hence, the macrophage is a very attractive target for designing therapeutic interventions[4], making it crucial to understand how cytokines regulate macrophage function in atherogenesis.

Cytokines of the type I interferon (IFN) family (IFNα and IFNβ) are produced by immune cells in response to pathogenic challenges such as viruses, bacteria and tumor cells. They induce antiviral responses[5,6], have immunomodulating activities and are clinically used to treat viral diseases, multiple sclerosis and certain tumors[5,7,8]. Recent data indicate that IFNβ is also necessary for sustaining TNF driven inflammation[9], promotes TNF-induced lethal shock[10] and mediates MHC-I induction by TNF[11]. In addition, constitutive low levels of type I IFN amplify IFNγ and interleukin-6 (IL-6) driven inflammatory responses[12,13]. Thus, type I IFNs also modulate immune responses that are not directly induced by pathogens. In sharp contrast to the clinical application of IFNβ as treatment for the pathologies mentioned above, type I IFNs have also been shown to promote disease in systemic lupus erythematosus (SLE), where inhibition strategies are being considered for therapy[14]. Interestingly, it was recently postulated that the increased risk of cardiovascular mortality in SLE patients[15] is linked to their elevated levels of type I IFNs[16]. Thus, depending on the context, type I IFN may have either disease promoting or disease inhibiting properties.

The important immunomodulatory activities of type I IFNs prompted us to hypothesize that they also have a role in atherogenesis. We show that IFNβ treatment induces chemokine dependent adhesion and migration of leukocytes and promotes atherosclerosis development *in vivo*. Consequently, we show that inhibition of type I IFN signaling in myeloid cells hampers cellular recruitment to lesions and thus atherosclerosis development. Finally, we found that type I IFN signaling is an integral feature of human atherosclerosis as well and is upregulated in plaque instability.

Results

IFNβ treatment of macrophages induces chemotactic mediators.

To study the effect of type I IFN on macrophages we first performed *in vitro* studies with cultured bone marrow derived macrophages (BMM) and analyzed cytokine secretion, uptake pathways and factors mediating adhesion and migration. While TNF and IL-12 were not induced by IFNβ, treatment did induce the production of the anti-inflammatory cytokine IL-10 (Figure 2.1A). Next, we analyzed uptake pathways by BMM that had been pretreated with IFNβ. Neither endocytosis of oxidatively modified LDL (oxLDL) nor phagocytosis of fluorescently labeled latex beads was affected by treatment of cells with IFNβ (Figure 2.1B and C). We further focused on molecules mediating attraction of cells to atherosclerotic lesions. FACS analysis of IFNβ treated macrophages showed no differences for any of the major integrins necessary for rolling and arrest at inflammatory sites (Figure 2.1D). Interestingly, gene expression of the chemokine and chemokine receptors (Figure 2.1E and F) CCL5 (RANTES) and its receptor CCR5, as well as CCR2 were upregulated in macrophages activated with IFNβ. However, further FACS analysis showed no significant upregulation of surface expression of both CCR2 and CCR5 (Figure 2.1G). In contrast, CCL5 secretion was strongly increased by activation of macrophages with IFNβ (Figure 2.1H). Next, we studied the role of the main receptor for type I IFN, IFNAR1, and therefore macrophages from mice with a myeloid specific deletion of IFNAR1[17] were used. Quantitative-PCR showed a deletion efficiency of IFNAR1 of 73.2% ± 1.4, and CCL5 induction by IFNβ was reduced to a similar extent in IFNAR1 deleted cells ($IFNAR1^{del}$) compared to control cells ($IFNAR1^{wt}$) (Figure 2.1I). Ablation of signal transducer and activator of transcription 1 (STAT1) signaling, as a key downstream transcription factor of type I IFN signaling, almost completely abolished induction of CCL5 by IFNβ (Figure 2.1J). CCL5 ELISA showed similar reduction in secreted CCL5 for $IFNAR1^{del}$ and $STAT1^{-/-}$ cells (data not shown). Treatment of macrophages with another type I IFN, IFNα showed similar effects on CCR5 and CCL5 (Supplemental figure S2.1). Thus, in addition to inducing IL-10, type I IFN treatment of primary macrophages induces chemotactic factors, which may influence leukocyte attraction and atherosclerosis development.

IFNβ treatment of macrophages enhances in vitro adhesion to endothelial cells and promotes leukocyte arrest at atherosclerosis prone sites in vivo.

In line with our data described above, we found that static adhesion of IFNβ treated macrophages to endothelial cells was enhanced (Figure 2.2A). Endothelial adhesion of macrophages lacking IFNAR1 or STAT1 was not inducible by IFNβ (Figure 2.2B and C). In line with a major role for CCL5-CCR5 signaling, we found that IFNβ-induced adhesion

was not affected in CCR1 deficient macrophages, but completely blunted in macrophages lacking CCR5 (Figure 2.2D). Absence of CCR2 showed an intermediate phenotype, with a moderate effect of IFNβ. *In vivo*, 4 days treatment of high fat fed apolipoprotein E deficient (*apoe*$^{-/-}$) mice with IFNβ led to a strong increase of leukocyte

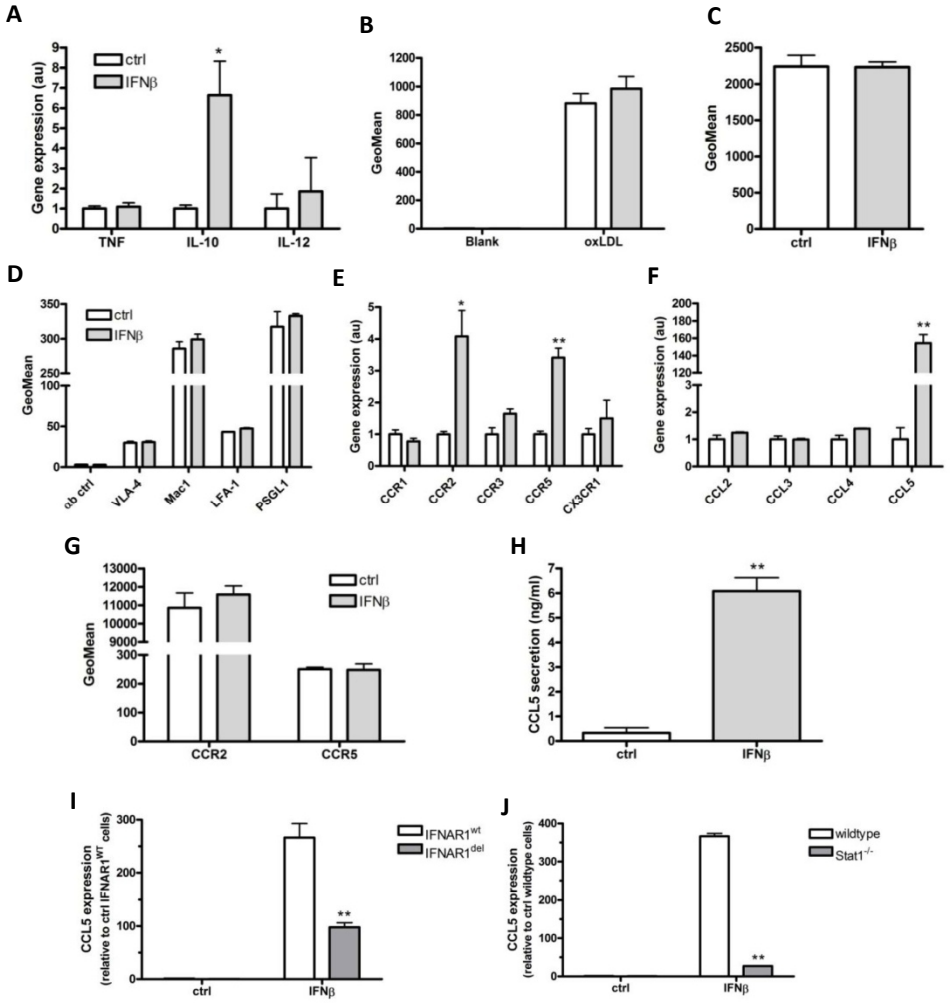

***Figure 2.1**: **The effect of IFNβ on cultured bone marrow derived macrophages.** (**A**) Treatment of macrophages with IFNβ induces IL-10 expression, without affecting TNF or IL-12. (**B**) Uptake of DiI-labelled oxLDL by control (ctrl) or IFNβ treated macrophages. (**C**) Uptake of fluorescent latex beads by control or IFNβ treated macrophages. (**D**) Surface expression of VLA-4, Mac1, LFA-1 and PSGL1 in control (ctrl) or IFNβ treated macrophages. (**E**) Relative gene expression of chemokine receptors in control or IFNβ treated macrophages. (**F**) Chemokine expression after IFNβ treatment of macrophages. (**G**) FACS analysis of CCR2 and CCR5 after treatment with IFNβ. (**H**) CCL5 secretion by macrophages after treatment with IFNß. (**I**) CCL5 expression in ctrl and IFNβ stimulated IFNAR1^{wt} and IFNAR1^{del} macrophages. (**J**) CCL5 expression in ctrl or IFNβ treated wildtype and STAT1$^{-/-}$ macrophages. Graphs are representative for at least two independent experiments. Bars represent mean of triplicate wells ± SEM (*P<0.05, **P<0.01).*

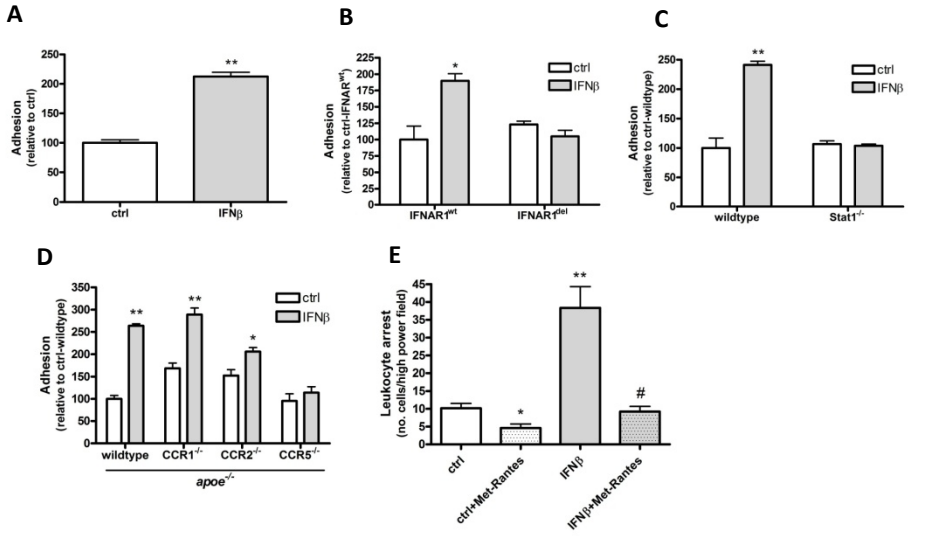

***Figure 2.2**: **IFN affects static in vitro adhesion and in vivo leukocyte arrest.** (**A**) Adhesion of untreated macrophages (ctrl) to endothelial cells compared to IFNβ treated macrophages (** P<0.01). (**B**) Adhesion of ctrl or IFNβ treated IFNAR1^{wt} and IFNAR1^{del} macrophages (* P<0.05). (**C**) Adhesion of ctrl or IFNβ treated wildtype or STAT1$^{-/-}$ macrophages (** P<0.01). (**D**) Adhesion of macrophages in ctrl or IFNβ treated CCR1$^{-/-}$, CCR2$^{-/-}$ or CCR5$^{-/-}$ macrophages, all on an apoe$^{-/-}$ background (* P<0.05, ** P<0.01). All bars for in vitro adhesion represent mean of triplicate wells ± SEM, stars indicate significant differences compared to ctrl treated cells of the same genotype. (**E**) Leukocyte arrest in the carotid artery of apoe$^{-/-}$ mice (ctrl), after treatment with Met-Rantes, after 4-days IFNβ treatment, or 4-days IFNβ treatment combined with Met-RANTES treatment (n=8, 4, 7 and 5 for the four groups respectively, error bars indicate SEM; *P<0.05 or **P<0.01 compared to control (ctrl), # P<0.01 compared to IFNβ).*

arrest in the carotid arteries of these animals (Figure 2.2E). Co-treatment of mice with the CCL5 antagonist Met-RANTES, just prior to analyzing leukocyte arrest, reduced leukocyte arrest in untreated mice and fully restored leukocyte adhesion to control levels in IFNβ treated animals (Figure 2.2E). Thus, IFNβ treatment *in vitro* and *in vivo* promotes leukocyte adhesion and attraction to the endothelium through CCL5-CCR5 dependent mechanisms.

IFNβ treatment of mice accelerates atherogenesis in apoe-/- and ldlr-/- mice.

Next, we analyzed the effect of IFNβ on atherogenesis in two well-established mouse models of atherosclerosis. Daily injections of IFNβ in a collar-induced atherosclerosis model in *apoe*$^{-/-}$ mice[18] did not alter plasma cholesterol levels (27.89±2.10 and 26.98±2.04 mM; for the ctrl and IFNβ treated, respectively) but increased plasma levels of IL-10 (3.90±2.1 and 19.21±5.8 pg/ml, p<0.05; for the ctrl and IFNβ treated,

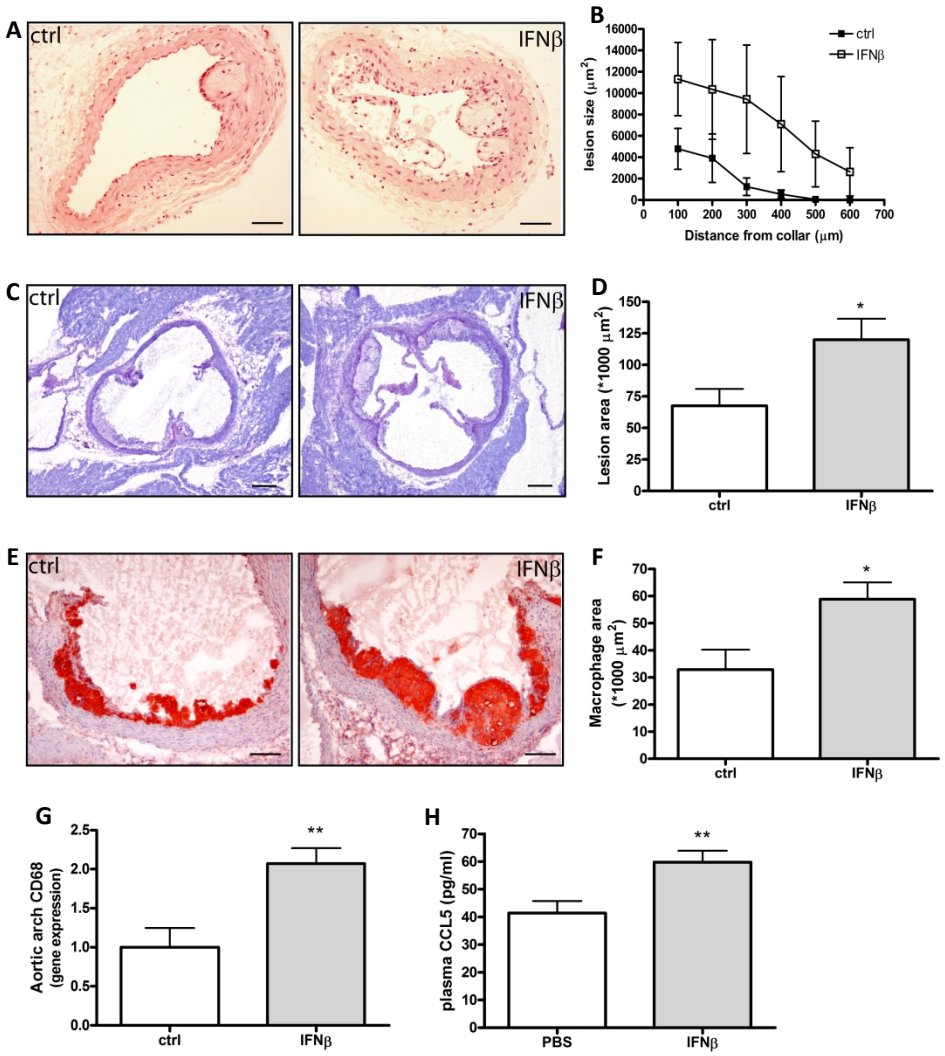

***Figure 2.3**: **IFNβ treatment accelerates atherosclerosis in apoe$^{-/-}$ and ldlr$^{-/-}$ mice.** (**A**) Representative lesions of control (ctrl) or IFNβ treated mice of collar induced atherosclerosis in apoe$^{-/-}$ mice. Scale bar indicates 50 µm. (**B**) Lesion area measured at six sequential locations proximal from the collar in apoe$^{-/-}$ mice which were control or IFNβ treated (** P<0.01 by two-way ANOVA; n=9/12). (**C**) Representative lesions in the aortic root of control (ctrl) or IFNβ treated ldlr$^{-/-}$ mice. Scale bar indicates 200 µm. (**D**) Lesion area at the aortic root of control or IFNβ treated ldlr$^{-/-}$ mice (*p<0.05; n=12/14). (**E**) Representative MOMA-2 stained lesions from control and IFNβ treated ldlr$^{-/-}$ mice. Scale bar indicates 100 µm. (**F**) Absolute macrophage area in lesions from control or IFNβ treated ldlr$^{-/-}$ mice (* P<0.05; n=12/14). (**G**) CD68 expression in aortic arches from control or IFNβ treated ldlr$^{-/-}$ mice (**p<0.01; n=11/14). (**H**) CCL5 levels in plasma from ctrl- or IFNβ-treated ldlr$^{-/-}$ mice (**p < 0.01; n=9/11). Shown are mean ± SEM.*

respectively). Atherosclerotic lesion analysis (Figure 2.3A and B) showed a strong increase in lesion formation in mice that had been treated with IFNβ. The same effect

was seen in low-density lipoprotein receptor deficient (*ldlr*$^{-/-}$) mice where IFNβ treatment increased aortic root atherosclerotic lesion size by almost 2-fold (Figure 2.3C and D) without any effects on plasma cholesterol (26.30±1.78 and 26.86±1.66; for the ctrl and IFNβ treated, respectively) or blood leukocyte levels (B220^{+} B-cells, CD3^{+} T-cells, Gr1^{hi}CD11b^{+} neutrophils and Gr1$^{int/-}$CD11b^{+} monocytes; data not shown). Staining for macrophage content showed an approximate two-fold increase in absolute macrophage area in IFNβ-treated mice (Figure 2.3E and F). Gene expression analysis of aortic arches from these mice also showed increased expression of the macrophage marker CD68 (Figure 2.3G), further indicating increased macrophage accumulation in atherosclerotic lesions. More extensive immunohistochemical analysis of the aortic root lesions did not show any significant difference with respect to neutrophil or T-cell accumulation, TUNEL positive cells or necrotic core formation (data not shown). Of interest, we did find an induction of circulating CCL5 levels in IFNβ-treated mice (Figure 2.3H). Thus, IFNβ treatment enhances atherosclerosis development, despite the induction of IL-10.

Myeloid deletion of IFNAR1 reduces atherosclerosis development and prevents macrophage and neutrophil accumulation in lesions.

Subsequently, we investigated whether endogenous production of type I IFN also contributes to atherosclerosis development. We first analyzed whether typical type I IFN signature genes were expressed in lesions of atherosclerotic mice. Indeed, expression of OAS1, OAS2, MX2 and IRF9 could be readily detected in aortic arches from *ldlr*$^{-/-}$ mice that had been fed a high fat diet for 6 or 9 weeks (supplemental figure S2.2), indicating that endogenous type I IFN signaling is present. We then performed a transplantation using bone marrow from IFNAR1^{wt} and IFNAR1^{del} mice, specifically lacking IFNAR1 in their myeloid lineage, to *ldlr*$^{-/-}$ mice to yield atherosclerosis susceptible mice that were either wildtype (*IFNAR1*^{wt}) or deleted (*IFNAR1*^{del}) for *IFNAR1* in their myeloid cells. Compared with *IFNAR1*^{wt} mice, mice lacking myeloid IFNAR1 showed a strong reduction in atherosclerotic lesion size (-34%) (Figure 2.4A and 4B) after 11 weeks of high fat diet. Lesions were mainly composed of macrophages (Figure 2.4C) and absolute macrophage area in the lesions was reduced in the *IFNAR1*^{del} mice (Figure 2.4D). Furthermore, the lesions were of intermediate phenotype, consisting mainly of macrophage derived foam cells with a fibrotic cap and some necrosis. *IFNAR1*^{del} mice showed reduced numbers of advanced lesions and increased moderate and early lesions (Figure 2.4E).

Plasma cholesterol levels did not differ between groups just before the start of the diet and after high fat feeding (Figure 2.4F). In addition, total leukocyte counts (Figure 2.4G) and blood levels of leukocyte subsets (Figure 2.4H) did not differ between *IFNAR1*^{wt} and *IFNAR1*^{del} mice. Plasma cytokine levels were reduced, with a strong reduction (-70%) of

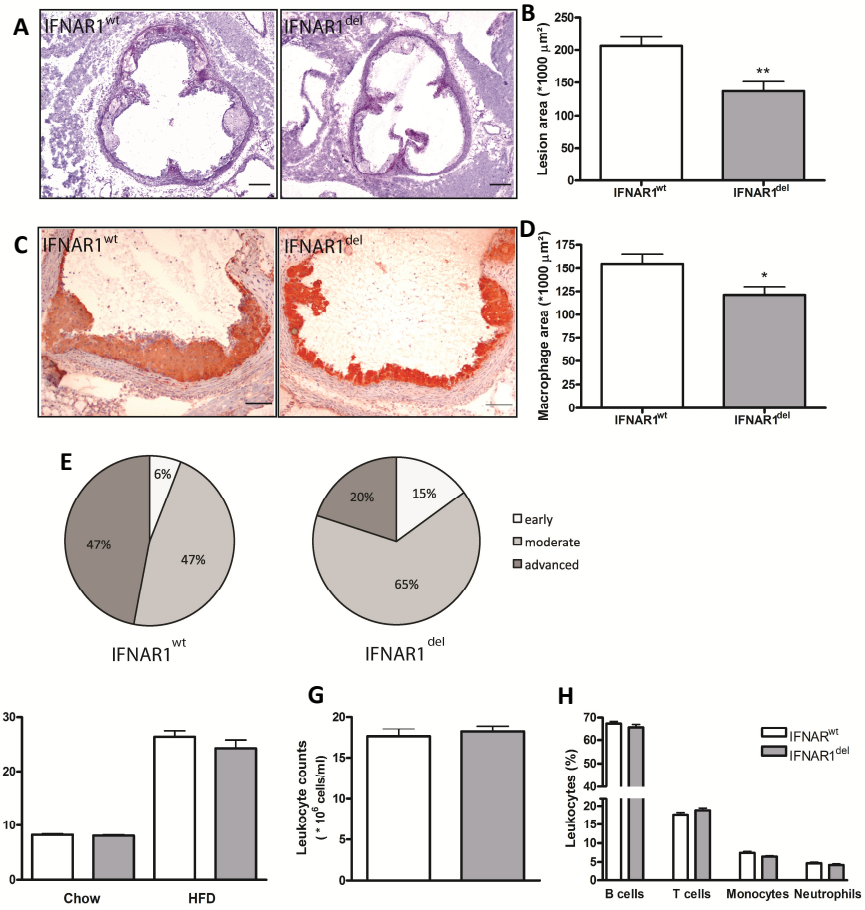

***Figure 2.4**: Absence of myeloid IFNAR1 reduces atherosclerosis development. Bone marrow from conditional mice devoid of IFNAR1 only on myeloid cells was transplanted to ldlr$^{-/-}$ mice to yield atherosclerosis susceptible mice that were either wildtype (IFNAR1^{wt}) or deleted (IFNAR1^{del}) for IFNAR1 in their myeloid cells. (**A**) Representative toluidin blue stained lesions from IFNAR1^{wt} and IFNAR1^{del} mice. Scale bar indicates 200 µm. (**B**) Lesion area at the aortic root of IFNAR1^{wt} and IFNAR1^{del} mice (** P<0.01; n=19/15). (**C**) Representative MOMA-2 stained lesions from IFNAR1^{wt} and IFNAR1^{del} mice. Scale bar indicates 100 µm. (**D**) Absolute macrophage area in lesions from IFNAR1^{wt} and IFNAR1^{del} mice (* P<0.05; n=17/14). (**E**) Lesion severity in IFNAR1^{wt} and IFNAR1^{del} mice (P<0.05 by Chi-square test; n=19/15). (**F**) Plasma cholesterol levels before (chow) and after 8 weeks of high fat diet (HFD) in IFNAR1^{wt} and IFNAR1^{del} transplanted mice. (**G, H**) Total leukocyte counts and relative levels of leukocyte subsets. Shown are mean ± SEM.*

circulating IL-6 (supplemental figure S2.2) and a borderline (P=0.05) reduction of the chemokine CCL2 (MCP-1) in *IFNAR1*^{del} mice.

Further examination of the lesions showed the neutrophil content to be decreased in atherosclerotic lesions of *IFNAR1*^{del} mice (Figure 2.5A and B), while T-cell content did

not differ (Figure 2.5C). Since type I IFNs have been implicated in the regulation of cell survival and proliferation[5], cell proliferation and apoptosis were assessed by Ki-67 and TUNEL staining, but no differences were observed between the two groups (Figure 2.5D and E). We did however find a strong (>70%) reduction of necrosis in the lesions of *IFNAR1*^{del} mice (Figure 2.5F and G), which is often considered a plaque destabilizing consequence of impaired efferocytosis in the lesions[19]. Thus, endogenously produced type I IFNs activate myeloid cells, increase lesional accumulation of macrophages and neutrophils, promote necrotic core formation, and thereby promote atherosclerosis development.

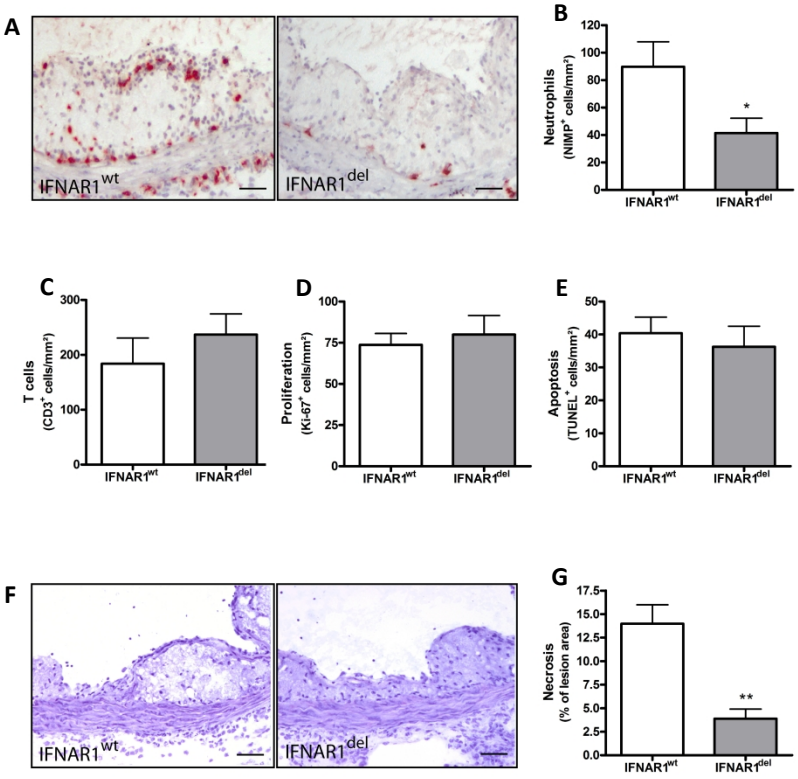

***Figure 2.5**: Absence of myeloid IFNAR1 influences the phenotype of atherosclerotic lesions. (**A**) Representative NIMP stainings of lesions from IFNAR1^{wt} and IFNAR1^{del} mice. Scale bar indicates 50 μm. (**B**) Neutrophil influx in lesions from IFNAR1^{wt} and IFNAR1^{del} mice (*P<0.05). (**C, D, E**) T-cells, proliferation and apoptosis in lesions from IFNAR1^{wt} and IFNAR1^{del} mice. (**F**) Representative examples of necrotic core in lesions from IFNAR1^{wt} and IFNAR1^{del} mice. Scale bar indicates 50 μm. (**G**) Necrosis in lesions from IFNAR1^{wt} and IFNAR1^{del} mice (** P<0.001). For all analyses, n=19 and 15 for IFNAR1^{wt} and IFNAR1^{del} mice respectively. Shown are mean ± SEM.*

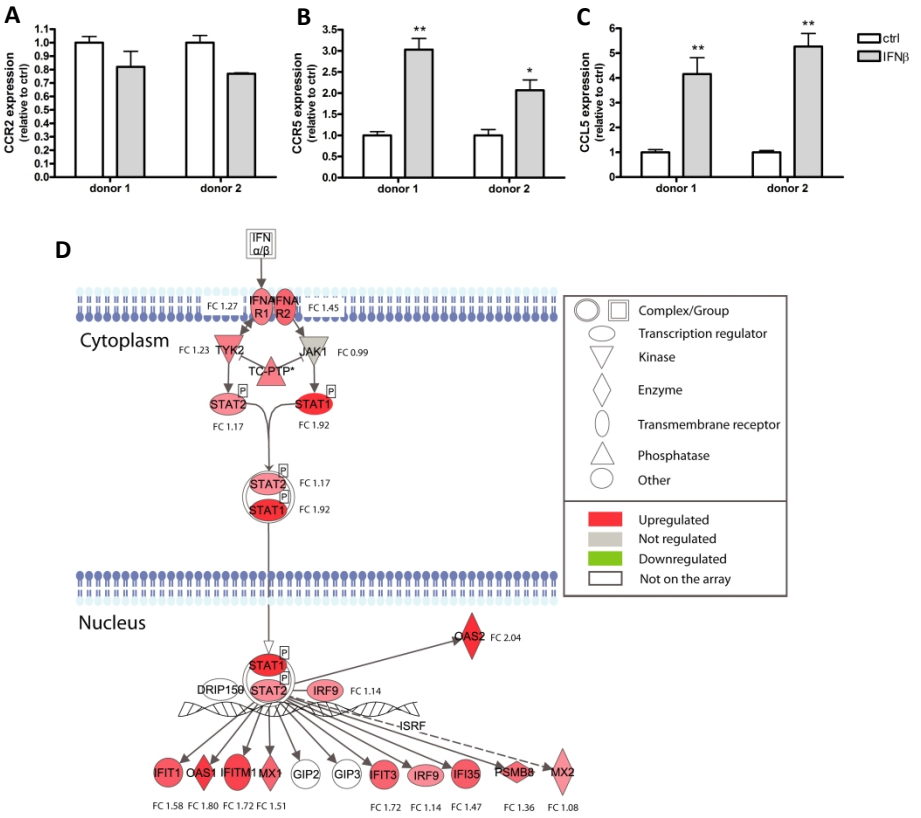

***Figure 2.6**: IFNβ induces chemotactic factors in human primary macrophages and type I IFN signaling is upregulated in unstable regions of human atherosclerotic lesions. (**A**) CCR2 expression in macrophages from two independent donors after IFNβ treatment. (**B**) CCR5 expression in macrophages from two independent donors after IFNβ treatment (*P<0.05; **P<0.01). (**C**) CCL5 expression in macrophages from two independent donors after IFNβ treatment (**P<0.01). Bars indicate mean of triplicate wells ± SEM. (**D**) Ingenuity Pathway Analysis of the differentially expressed genes in stable sections compared to ruptured sections of carotid endarterectomy specimens. Red signals indicate upregulation and the pathway showed a strongly significant (P=2.36*10^{-6}, ratio 16/23 (0.696)) upregulation of type I IFN signalling. Indicated are the fold-changes (FC) of the respective genes.*

Type I IFN induces chemotactic mediators in human macrophages and type I IFN signaling is associated with plaque instability in human atherosclerotic lesions.

To investigate whether type I IFNs also play a role in the pathogenesis of human atherosclerosis, we first analyzed the effect of IFNβ on primary human macrophages. Although IFNβ did not induce CCR2 (Figure 2.6A), it did increase expression of both CCR5 and CCL5 (Figure 2.6B and C), and induced secretion of CCL5 as analyzed by ELISA (Supplemental Figure S2.3), confirming our mouse data. Next, Ingenuity Pathway Analysis was performed on an expression database of human atherosclerotic lesions.

We compared data from stable carotid endarterectomy specimens and ruptured specimens, which had been classified according to the Virmani-classification[20]. Interestingly the type I IFN pathway showed a highly significant upregulation in ruptured lesions when compared to stable lesions (Figure 2.6D). In addition to the upregulation of the type I IFN pathway, also several chemotactic factors including CCR5 and CCL5 were upregulated in ruptured atherosclerotic lesions (Supplemental Table ST2.1). These data show that type I IFN signaling correlates with plaque rupture in human atherosclerosis.

Discussion

In this paper we show that treatment with IFNβ enhances atherogenesis in different models and that myeloid specific inhibition of type I IFN signaling reduces atherosclerosis development. We demonstrate that IFNβ treatment induces chemotactic factors and thereby promotes leukocyte attraction to atherosclerosis prone sites. Accordingly, absence of endogenous myeloid type I IFN signaling reduced accumulation of cells from the myeloid lineage in the lesions. Thus, type I IFNs are pro-atherosclerotic cytokines that act by promoting chemokine dependent leukocyte recruitment to atherosclerotic lesions. Interestingly, upregulation of type I IFN signaling is also associated with plaque instability in human atherosclerotic lesions.

The family of type I IFNs consists of many members with IFNα and IFNβ being the most abundant and best studied. It was previously shown that IFNα treatment promotes atherosclerosis in *ldlr*$^{-/-}$ mice. However, this effect was accompanied by elevated plasma levels of triglycerides and cholesterol[21], which complicates the interpretation of these data. Therefore, we chose to use IFNβ in our treatment studies, although we found that IFNα had similar *in vitro* effects on chemotactic factors. Zhang et al. describe that IFNβ administration attenuates lesion formation induced by carotid artery ligation in angiotensin-II infused *apoe*$^{-/-}$ mice [22], which is in contrast to our findings. Thus, angiotensin acceleration of atherosclerosis depends on other inflammatory factors than our models. We now show, using two different models of atherosclerosis, that IFNβ treatment promotes atherosclerosis in high fat fed mice. In both a collar-accelerated model (in the *apoe*$^{-/-}$ mice) and a solely hyperlipidemia-driven model (in the *ldlr*$^{-/-}$ mice), atherogenesis was enhanced by IFNβ, without influencing plasma lipid parameters.

We found that IFNβ treatment of isolated primary macrophages induced IL-10. In addition, IFNβ treatment of mice led to increased levels of circulating IL-10, similarly as is observed in MS patients and mouse models for MS[23]. IL-10 is a classical anti-inflammatory cytokine and the beneficial effects of IFNβ treatment in relapsing remitting MS patients is at least partly attributed to anti-inflammatory factors it induces[24]. The induction of this anti-atherogenic[25] cytokine contrasts with the atherosclerosis promoting effect that we observe for IFNβ. However, we did find IFNβ

to specifically elicit chemotactic factors with a reported pro-atherogenic function. CCL2 (MCP-1) and CCL5 (RANTES) and their receptors (CCR2 and CCR5, respectively) are important in regulating attraction of cells to atherosclerotic lesions and thereby control atherosclerosis development[26-28]. We found that especially CCL5 was strongly upregulated after IFNβ treatment of macrophages, coinciding with a modest induction of CCR5 and CCR2. CCL5 expression after IFNβ was reduced in macrophages lacking either IFNAR1 or STAT1, indicating that CCL5 is either a direct target of IFNAR1-STAT1 signaling or regulated by interferon regulatory factors (IRFs) induced by STAT1 activation[29-31]. We could also show that IFNβ increased static adhesion of macrophages to endothelial cells, again in an IFNAR1 and STAT1 dependent manner, which was abolished by macrophage CCR5 deficiency, but not by deficiency for CCR1, the other major receptor for CCL5. CCR2 deficiency had a modest effect on IFNβ induced macrophage adhesion. Furthermore, enhanced leukocyte arrest induced by IFNβ in *apoe*$^{-/-}$ mice could be completely blocked by co-treatment with the CCL5-receptor inhibitor, Met-RANTES. Therefore we think that despite the induction of IL-10 by IFNβ, the atherosclerotic process is actually enhanced through the induction of especially the CCL5-CCR5 axis, thereby promoting attraction of leukocytes to lesions.

Type I interferons are massively produced by various cell types in response to viruses and other microbial stimuli, through engagement of toll-like receptors such as TLR3, TLR4 and TLR9. Major *in vivo* sources of type I IFN are plasmacytoid dendritic cells (pDCs). Interestingly, DCs and more specifically pDCs have been identified in human atherosclerotic lesions and have been associated with rupture prone areas of the lesions[32-34]. Using datasets from stable and ruptured human endarterectomy segments we now show that segments of ruptured plaque have an upregulation of the type I IFN signaling pathway, when compared to stable segments. Thus, ruptured sections of atherosclerotic lesions show induction of type I IFN signaling. This may consequently increase attraction of inflammatory cells, further contributing to matrix degradation and plaque destabilization.

Using conditional knockouts we demonstrate that myeloid cells are important targets for endogenously produced type I IFN in atherosclerosis. Through induction of chemotactic factors, type I IFN signaling promotes macrophage adhesion to endothelial cells. As such, deletion of IFNAR1 in myeloid cells ultimately leads to reduced accumulation of macrophages in atherosclerotic lesions and thereby reduces atherosclerosis development. We determined deletion efficiency to be approximately 70% in our bone marrow cultures, indicating some remaining type I IFN signaling. In addition, it was previously shown that circulating monocytes from LysMCre-IFNAR1^{floxed} mice show severely hampered but not fully absent type I IFN signaling[17]. Thus, the phenotype we observe is the likely result of reduced but not completely absent myeloid type I signaling. Whether this merely means an underestimation of the contribution of endogenous type I signaling in atherosclerosis or whether additional function are therefore undetectable remains to be discovered.

In addition to affecting macrophages, myeloid IFNAR1 deficiency also reduces accumulation of neutrophils in the lesions, which have recently been found to contribute to atherosclerosis[35,36]. Disturbed macrophage CCL5 production may impair lesional recruitment of neutrophils, which use CCR1 to respond to CCL5[37]. Alternatively, IFNAR1 deficiency may have unexplored direct effects on neutrophils influencing migration to inflammatory sites. However, in our treatment approach with *ldlr*$^{-/-}$ mice, neutrophil accumulation in the lesions was not affected (data not shown), indicating that IFNβ treatment does not affect neutrophils in these studies. In addition, although we clearly identify that myeloid IFNAR1 signaling highly controls lesion development, we cannot exclude that other targets of type I IFN, such as endothelial cells or fibroblasts, will contribute to the effects that we observe upon treatment of the mice with IFNβ.

We found that inhibition of endogenous myeloid type I IFN signaling reduces necrotic core formation and thus directly affects plaque stability. Reduced necrosis paralleled impaired progression of the lesions. However, upon analysis of a subset of lesions from both groups with similar size (84.5±4.8 and 81.4±4.2 x1000 μm^2 for the *IFNAR1*^{wt} and *IFNAR1*^{del} groups) and severity (moderate lesions), we found that necrotic core formation was still reduced in the *IFNAR1*^{del} group (12.6±2.4% and 4.0±1.3% for the *IFNAR1*^{wt} and *IFNAR1*^{del} groups). Thus, inhibition of myeloid IFNAR1 function directly inhibits necrotic core formation independent of lesion size and severity and without any clear effects on endocytosis or phagocytosis. Depending on the experimental context, both pro- and anti-cell death functions have been attributed to type I IFN. The antitumor effects of type I IFN are at least partly attributed to the induction of cell death by type I IFN[5], in which IFNs and interferon stimulated genes are both sensitizing tumor cells to immune cell mediated cytotoxicity and are augmenting lytic activity of immune cells. In line, several groups have reported that endogenous type I interferon signaling sensitizes cells to pathogen induced cell death[38,39] or apoptosis induced by serum deprivation[40]. In addition, treatment of MS patients with IFNβ was shown to prime monocyte derived macrophages for apoptotic cell death[41]. We show that inhibition of myeloid type I IFN signaling reduces necrosis, but treatment of *ldlr*$^{-/-}$ mice with IFNβ did not enhance necrotic core formation. Thus, in atherogenesis the necrosis promoting effect of endogenous type I IFN is apparently already maximal and not further promoted by exogenously added IFNβ.

Our findings are supported by previous studies demonstrating that type I IFNs are important inducers of cellular migration. The attraction of inflammatory cells is an important feature of type I IFN action in fighting microbial infections[5]. In addition, it was recently shown that type I IFNs are also essential mediators of TNF-induced lethal inflammatory shock by enhancing cell death and promoting white blood cell influx in tissues through induction of a set of chemokines[10]. Moreover, in search of IFNβ related adverse side effects in MS patients several groups performed gene expression studies on peripheral blood mononuclear cells and demonstrated induction of chemotactic

factors in response to IFNβ treatment[42,43]. In line with this it was recently shown that a group of chemotactic genes, including CCL5 and CCL2, was among the most prominently upregulated ones in brain macrophages (microglia) treated with IFNβ [17]. We focused on the major chemokines and chemokine receptors with a demonstrated role in atherogenesis and found that especially the CCL5-CCR5 axis may be very important in mediating the pro-atherogenic function of IFNβ.

In conclusion, we show that type I IFNs promote atherosclerosis development. Contrary to the antiviral effect of type I IFN acting on a multitude of cell types, we report that the specific interaction of endogenous type I IFN with myeloid cells enhances the recruitment of these cells to atherosclerotic lesions. This effect is in accordance with an observed type I IFN induced macrophage adhesion mediated by upregulation of specific chemokines and their receptors, mainly involving the CCL5-CCR5 axis. Furthermore, we present plaque instability as a direct consequence of myeloid IFN signaling. Collectively, these data raise the demand for further detailed analyses of cardiovascular risk in patients treated with type I IFN and may even imply that caution should be taken in using type I IFN as a therapeutic option. In contrast, targeting of type I IFN signaling may be an attractive target for prevention and treatment of atherosclerosis.

Experimental Procedures

Mice

C57BL/6 mice and *ldlr*$^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME), *apoe*$^{-/-}$ mice on a C57BL/6 background were obtained from Iffa Credo (Lyon, France). *IFNAR1*$^{fl/fl}$, *LysMCre-IFNAR1*$^{fl/fl}$, *apoe*$^{-/-}$, *apoe*$^{-/-}$*CCR1*$^{-/-}$, *apoe*$^{-/-}$*CCR2*$^{-/-}$, *apoe*$^{-/-}$*CCR5*$^{-/-}$ and *STAT1*$^{-/-}$ mice were all on a C57BL/6 background and have been described before[17,44-46]. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University or complied with German animal protection law.

Interferons

Recombinant murine IFNα or IFNβ were obtained from Hycult Biotec (Uden, The Netherlands). Human IFNβ was obtained from Peprotech (Rocky Hill, NJ, USA).

In vitro murine macrophage culture

Bone marrow cells were isolated from femurs and tibiae of wildtype mice (C57BL/6), *IFNAR1*$^{fl/fl}$, *LysMCre-IFNAR1*$^{fl/fl}$, *apoe*$^{-/-}$, *apoe*$^{-/-}$*CCR1*$^{-/-}$, *apoe*$^{-/-}$*CCR2*$^{-/-}$, *apoe*$^{-/-}$*CCR5*$^{-/-}$ or *STAT1*$^{-/-}$ mice. Cells were cultured in RPMI-1640 (Gibco Invitrogen, Breda, The Netherlands) with 10% heat inactivated fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 ug/ml), L-glutamine 2 mM (all Gibco Invitrogen, Breda, The Netherlands) (R10) supplemented with 15% L929-

conditioned medium (LCM) for 8-9 days to generate bone marrow derived macrophages (BMM), as described previously[47]. Cells were treated for 24h with 100U/ml IFNα or IFNβ.

In vitro human macrophage culture

Human mononuclear cells were obtained by apheresis of 2x the blood volume from healthy volunteers using a Cobe® Spectra (CaridianBCT Europe, Zaventhem, Belgium). Monocytes were subsequently enriched by counter flow centrifugation using the Elutra Cell Separation System (CaridianBCT) and were cultured in R10 supplemented with 5ng/ml M-CSF (Peprotech) for 8 days to generate human macrophages. Cells were treated for 24h with 100U/ml human IFNβ.

Gene expression

RNA was isolated from BMM or human macrophages with the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). 500 ng total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative PCR was performed using 10 ng cDNA, 300nM of each primer, IQ SYBR Green Supermix (BioRad) in a total volume of 20 µl. Deletion efficiency in BMM of the LysMCre-IFNAR1$^{fl/fl}$ mice was measured by Q-PCR on 25ng DNA as described before[47], using primers specifically detecting the floxed and not the deleted or wildtype IFNAR1 allele. All gene expression levels were corrected for cyclophilin A as house keeping gene and primer sequences are available upon request.

In vitro oxLDL and bead uptake

BMM untreated or treated for 24h with 100U/ml IFNβ were incubated for 3h in Optimem-1 with fluorescently labeled latex beads or 25 µg/ml DiI labeled oxLDL, generated through copper oxidation, as described previously[47]. Uptake was assessed by flow cytometry after residual beads or oxLDL were washed away.

Flow cytometry

BMM (+/- 100U/ml IFNβ for 24h) were stained with antibodies against VLA-4 (Cedarlane, Burlington, Ontario, Canada), Mac1, LFA-1, PSGL1 (all BD Pharmingen, Erembodegem, Belgium), CCR2 (Epitomics, Burlingame, CA, USA) or CCR5 (BD Pharmingen) and their mean fluorescence was measured by FACS analysis. For the CCR2 staining, cells were permeabilized using Cytoperm™ Plus Permeabilization Buffer and Perm/Wash™ Buffer (both BD Pharmingen).

ELISA

Murine CCL5 secretion from BMM (+/- 100U/ml IFNβ for 24h) was measured by ELISA using anti-mouse CCL5 (R&D Systems, Abigndon, UK) as coating antibody and biotinylated anti-mouse CCL5 (R&D Systems) as detection antibody with mouse CCL5

(Peprotech) as standard. Human CCL5 secretion in the supernatants from human macrophages (+/- 100U/ml IFNβ for 24h) was measured by commercial ELISA (PBL Interferon Source, New Brunswick, NJ, USA). Absorbance was measured at 450nm using a microtiterplate reader (BioRad, Hercules, CA, USA).

In vitro adhesion assay

A confluent monolayer of bEND5 endothelial cells was grown in fluorescence 96 wells microplates (Greiner Bio-one, Frickenhausen, Germany). Triplicate wells were incubated for 30 min with 10^5 BMM (+/- 100U/ml IFNβ for 24h) that had been fluorescently labeled with a PKH dye according to the manufacturer's instructions (Sigma Aldrich, Zwijndrecht, The Netherlands). Subsequently, the wells were washed three times with R10 and adherent cells were measured by fluorometry in a Synergy HT microtiter plate reader (BioTek, Bad Friedrichshall, Germany) at an excitation of 485nm and an emission of 520nm.

In vivo leukocyte adhesion

Apoe$^{-/-}$ mice were put on a high fat diet (0.15% cholesterol, 21% fat, Altromin) for 6 weeks and during the last 4 days daily injected subcutenously with either saline or IFNβ (5000U). Four mice receiving saline injections and five receiving IFNβ injections were co-treated with an intravenous Met-RANTES (50 μg/mouse) injection 30 min before the experiment. After sedation (intraperitoneal Ketamine/Xylazin) and intravenous rhodamin injection, the left carotid artery was exposed and 3 high power fields (hpf) near the carotid bifurcation were visualized by epifluorescence microscopy (Zeiss Axiotech, 20x water immersion objective) as described before[48]. Short movies were recorded for each hpf, in which the cells attaching to the vessel wall were counted by eye.

Collar-induced atherosclerosis

Seventeen-week-old male *apoe*$^{-/-}$ mice (n=21) were operated after 3 weeks of high fat diet (0.25% cholesterol, 16% fat) to introduce a 2mm long non-constrictive silastic tube around both carotid arteries, as described before[18]. During the 24 days post-operation, the high-fat diet was continued and the mice were treated by daily subcutaneous injection of either IFNβ (5000U) or saline. Upon sacrifice the right carotid artery was isolated and embedded in paraffin. Sections of 5μm were made and after every 100μm, sections were stained with haematoxilin/eosin for lesion area analysis.

IFNβ treatment in ldlr-/- mice

Twelve-week-old male *ldlr*$^{-/-}$ mice were fed a high fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 6 weeks in order to induce early atherogenesis. At this point, daily subcutaneous injections of either IFNβ (5000U) or saline were started and continued for 3 weeks while the high fat diet was continued. Upon sacrifice the heart and aorta were taken out. The hearts were cut perpendicular to the heart axis just

below the atrial tips. Tissue was frozen in tissue-tec (Shandon, Veldhoven, The Netherlands) and cut into sections of 7μm as described before[47]. Serial cross-sections from every 42μm were stained with toluidin blue. All lesion areas were quantified using Adobe Photoshop software. The aortas were snap-frozen and RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) including DNAse treatment. Gene expression was assessed by Q-PCR, similarly as described above.

Bone marrow transplantation

One week before transplantation, female *ldlr*$^{-/-}$ mice were housed in filter top cages and provided with acidified water containing neomycin (100mg/l; Gibco, Breda, The Netherlands) and polymyxin B sulphate (6x10^4 U/l; Gibco). The animals received two times 6Gy total body irradiation on two consecutive days. On the second day, bone marrow was isolated from 6 LysMCre-IFNAR1$^{fl/fl}$ mice (IFNAR1^{del}) and 6 IFNAR1$^{fl/fl}$ littermates (IFNAR1^{wt}) and 10^7 cells/mouse were injected intravenously to rescue the hematopoietic system of the irradiated mice. Four weeks after the transplantation, mice were fed a high fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 11 weeks. After sacrifice, the hearts from the bone marrow transplanted mice were taken out and lesion size in the aortic root was measured as described above. The lesions were also typed according to severity as early, moderate and advanced as described before[47].

Mouse blood parameters

At several time points during all *in vivo* atherosclerosis experiments, blood was drawn from the mice. Plasma lipid levels were monitored enzymatically (Sigma Aldrich, Zwijndrecht, The Netherlands) and plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array kit (BD-Pharmingen, San Diego). For the bone marrow transplanted mice, leukocytes were counted using a Coulter counter and blood cell distribution was quantified by flow cytometry after antibody staining with either Mac1-PE and Gr1-FITC for macrophages and granulocytes or 6B2-PE and KT3-FITC for B- and T-cells (BD-Pharmingen, Erembodegem, Belgium).

Immunohistochemical staining

Lesions from the aortic root were fixed in acetone and incubated with antibodies against macrophages (MOMA-2, a gift from G. Kraal), granulocytes (NIMP, directed against Ly6G, a gift from P. Heeringa), T-cells (KT3, directed against CD3, a gift from G. Kraal) and proliferating cells (Ki-67, Dako, Glostrup, Denmark). Apoptotic cells in the plaques were stained by the TUNEL staining (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. Necrotic areas were analyzed on toluidin blue stained sections and identified by the presence of pyknosis, karyorrhexis or complete absence of nuclei.

Human plaque transcriptomics

Microarray analysis was performed on RNA isolated from 44 (22 stable and 22 ruptured) human carotid plaque specimens using Illumina Human Sentrix-8 V2.0 BeadChip® technology to detect differential expression. For pathway analysis we used the Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) system.

Statistical analysis

The statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between 2 groups were evaluated using a *t*-test, unless stated otherwise. Values are represented as mean ± SEM. A *P* value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

References

1. Weber, C., Zernecke, A. & Libby, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* **8**, 802-815 (2008).
2. Hansson, G.K. & Libby, P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* **6**, 508-519 (2006).
3. Tedgui, A. & Mallat, Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* **86**, 515-581 (2006).
4. Li, A.C. & Glass, C.K. The macrophage foam cell as a target for therapeutic intervention. *Nat Med* **8**, 1235-1242 (2002).
5. Borden, E.C.*, et al.* Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* **6**, 975-990 (2007).
6. Katze, M.G., He, Y. & Gale, M., Jr. Viruses and interferon: a fight for supremacy. *Nat Rev Immunol* **2**, 675-687 (2002).
7. Paty, D.W. & Li, D.K. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology* **43**, 662-667 (1993).
8. Takaoka, A. & Yanai, H. Interferon signalling network in innate defence. *Cell Microbiol* **8**, 907-922 (2006).
9. Yarilina, A., Park-Min, K.H., Antoniv, T., Hu, X. & Ivashkiv, L.B. TNF activates an IRF1-dependent autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I interferon-response genes. *Nat Immunol* **9**, 378-387 (2008).
10. Huys, L.*, et al.* Type I interferon drives tumor necrosis factor-induced lethal shock. *J Exp Med* **206**, 1873-1882 (2009).
11. Leeuwenberg, J.F., van Damme, J., Jeunhomme, G.M. & Buurman, W.A. Interferon beta 1, an intermediate in the tumor necrosis factor alpha-induced increased MHC class I expression and an autocrine regulator of the constitutive MHC class I expression. *J Exp Med* **166**, 1180-1185 (1987).
12. Mitani, Y.*, et al.* Cross talk of the interferon-alpha/beta signalling complex with gp130 for effective interleukin-6 signalling. *Genes Cells* **6**, 631-640 (2001).
13. Takaoka, A.*, et al.* Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. *Science* **288**, 2357-2360 (2000).
14. Ronnblom, L., Eloranta, M.L. & Alm, G.V. The type I interferon system in systemic lupus erythematosus. *Arthritis Rheum* **54**, 408-420 (2006).
15. McMahona, M. & Hahna, B.H. Atherosclerosis and systemic lupus erythematosus: mechanistic basis of the association. *Curr Opin Immunol* **19**, 633-639 (2007).
16. Lee, P.Y.*, et al.* Type I interferon as a novel risk factor for endothelial progenitor cell depletion and endothelial dysfunction in systemic lupus erythematosus. *Arthritis Rheum* **56**, 3759-3769 (2007).
17. Prinz, M.*, et al.* Distinct and Nonredundant In Vivo Functions of IFNAR on Myeloid Cells Limit Autoimmunity in the Central Nervous System. *Immunity* **28**, 675-686 (2008).
18. von der Thusen, J.H., van Berkel, T.J. & Biessen, E.A. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation* **103**, 1164-1170 (2001).
19. Tabas, I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol* **25**, 2255-2264 (2005).

20. Virmani, R., Kolodgie, F.D., Burke, A.P., Farb, A. & Schwartz, S.M. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* **20**, 1262-1275 (2000).
21. Levy, Z.*, et al.* Low-dose interferon-alpha accelerates atherosclerosis in an LDL receptor-deficient mouse model. *Eur J Intern Med* **14**, 479-483 (2003).
22. Zhang, L.N.*, et al.* Interferon-beta attenuates angiotensin II-accelerated atherosclerosis and vascular remodeling in apolipoprotein E deficient mice. *Atherosclerosis* **197**, 204-211 (2008).
23. Rudick, R.A.*, et al.* In vivo effects of interferon beta-1a on immunosuppressive cytokines in multiple sclerosis. *Neurology* **50**, 1294-1300 (1998).
24. Billiau, A. Anti-inflammatory properties of Type I interferons. *Antiviral Res* **71**, 108-116 (2006).
25. Mallat, Z.*, et al.* Protective role of interleukin-10 in atherosclerosis. *Circ Res* **85**, e17-24 (1999).
26. Braunersreuther, V.*, et al.* Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* **27**, 373-379 (2007).
27. Veillard, N.R.*, et al.* Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* **94**, 253-261 (2004).
28. Boring, L., Gosling, J., Cleary, M. & Charo, I.F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**, 894-897 (1998).
29. Cremer, I., Ghysdael, J. & Vieillard, V. A non-classical ISRE/ISGF3 pathway mediates induction of RANTES gene transcription by type I IFNs. *FEBS Lett* **511**, 41-45 (2002).
30. Melchjorsen, J., Sorensen, L.N. & Paludan, S.R. Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. *J Leukoc Biol* **74**, 331-343 (2003).
31. Taniguchi, T. & Takaoka, A. A weak signal for strong responses: interferon-alpha/beta revisited. *Nat Rev Mol Cell Biol* **2**, 378-386 (2001).
32. Bobryshev, Y.V. & Lord, R.S. Ultrastructural recognition of cells with dendritic cell morphology in human aortic intima. Contacting interactions of Vascular Dendritic Cells in athero-resistant and athero-prone areas of the normal aorta. *Arch Histol Cytol* **58**, 307-322 (1995).
33. Niessner, A.*, et al.* Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. *Circulation* **114**, 2482-2489 (2006).
34. Yilmaz, A.*, et al.* Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques. *Atherosclerosis* **176**, 101-110 (2004).
35. van Leeuwen, M.*, et al.* Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR-/- mice. *Arterioscler Thromb Vasc Biol* **28**, 84-89 (2008).
36. Zernecke, A.*, et al.* Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circ Res* **102**, 209-217 (2008).
37. Liehn, E.A.*, et al.* Ccr1 deficiency reduces inflammatory remodelling and preserves left ventricular function after myocardial infarction. *J Cell Mol Med* **12**, 496-506 (2008).
38. O'Connell, R.M.*, et al.* Type I interferon production enhances susceptibility to Listeria monocytogenes infection. *J Exp Med* **200**, 437-445 (2004).
39. Qiu, H.*, et al.* Type I IFNs enhance susceptibility to Chlamydia muridarum lung infection by enhancing apoptosis of local macrophages. *J Immunol* **181**, 2092-2102 (2008).
40. Wei, J., Sun, Z., Chen, Q. & Gu, J. Serum deprivation induced apoptosis in macrophage is mediated by autocrine secretion of type I IFNs. *Apoptosis* **11**, 545-554 (2006).

41. Van Weyenbergh, J., Wietzerbin, J., Rouillard, D., Barral-Netto, M. & Liblau, R. Treatment of multiple sclerosis patients with interferon-beta primes monocyte-derived macrophages for apoptotic cell death. *J Leukoc Biol* **70**, 745-748 (2001).
42. Satoh, J., Nanri, Y., Tabunoki, H. & Yamamura, T. Microarray analysis identifies a set of CXCR3 and CCR2 ligand chemokines as early IFNbeta-responsive genes in peripheral blood lymphocytes in vitro: an implication for IFNbeta-related adverse effects in multiple sclerosis. *BMC Neurol* **6**, 18 (2006).
43. Wandinger, K.P.*, et al.* Complex immunomodulatory effects of interferon-beta in multiple sclerosis include the upregulation of T helper 1-associated marker genes. *Ann Neurol* **50**, 349-357 (2001).
44. Durbin, J.E., Hackenmiller, R., Simon, M.C. & Levy, D.E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84**, 443-450 (1996).
45. Schober, A.*, et al.* Crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets. *Circ Res* **95**, 1125-1133 (2004).
46. Zernecke, A.*, et al.* Deficiency in CCR5 but not CCR1 protects against neointima formation in atherosclerosis-prone mice: involvement of IL-10. *Blood* **107**, 4240-4243 (2006).
47. Kanters, E.*, et al.* Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* **112**, 1176-1185 (2003).
48. Bernhagen, J.*, et al.* MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* **13**, 587-596 (2007).

Chapter 3

Myeloid IκBα deficiency promotes atherogenesis by enhancing leukocyte recruitment to the plaques

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Abstract

Activation of the transcription factor NF-κB appears to be involved in different stages of atherogenesis. In this paper we investigate the role of NF-κB inhibitor IκBα in atherosclerosis. Myeloid-specific deletion of IκBα results in larger and more advanced lesions in LDL-R-deficient mice without affecting the compositional phenotype of the plaques or systemic inflammatory markers in the plasma. We show that IκBα-deleted macrophages display enhanced adhesion to an *in vitro* endothelial cell layer, coinciding with an increased expression of the chemokine CCL5. Also, *in vivo* we found that IκBα^{del} mice had more leukocytes adhering to the luminal side of the endothelial cell layers that cover the atherosclerotic plaques. Moreover, we introduce ER-MP58 in this paper as a new immunohistochemical tool for quantifying newly recruited myeloid cells in the atherosclerotic lesion. This staining confirms that in IκBα^{del} mice more leukocytes are attracted to the plaques. In conclusion, we show that IκBα deletion in myeloid cells promotes atherogenesis, probably through an induced leukocyte recruitment to plaques.

Introduction

NF-κB is a transcription factor that translates the inflammatory stimuli from the environment into gene expression patterns regulating cell differentiation, activation, proliferation and apoptosis as well as the production of a set of inflammatory mediators. It is activated in response to pathogen detection by Toll-like receptor signaling or, in the inflammatory milieu, through different cytokine receptors such as the TNF receptor. Also, non-pathogen related activation, called "sterile inflammation", including stimuli such as free radicals, radiation and modified lipoproteins, can trigger NF-κB[1,2].

Rather than one transcription factor, NF-κB is in fact a family of homo- and heterodimers, with different possible combinations of the Rel-domain containing proteins NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel. In the absence of an activating stimulus, the NF-κB dimer is kept cytoplasmic because its nuclear localization signal is covered by an inhibitor belonging to the IκB family[3]. The IκB family consists of IκBα, IκBβ, IκBγ, IκBε and Bcl-3[4]. Following a signaling cascade initiated by an inflammatory stimulus, IκB is phosphorylated by a complex consisting of IκB kinase 1 (IKK1 or IKKα), IKK2 (or IKKβ) and NEMO (or IKKγ). This phosphorylation leads to ubiquitination and subsequent proteasomal degradation of the IκB, leaving NF-κB free to translocate to the nucleus[5]. Being NF-κB target genes themselves, the IκB family members are part of a negative feedback loop, retracting NF-κB from the nucleus back into the cytoplasm and thereby preventing excessive and irreversible NF-κB activation[6].

NF-κB activation is an important response in different infectious as well as non-infectious pathologies. Also in the different stages of atherogenesis, from early endothelial activation to eventual plaque rupture, NF-κB has been described as a key regulator[7,8]. Atherosclerosis is a slowly progressing, chronic inflammatory disease of the large arteries representing the most common cause of death in western society[9]. This process is initiated when modified lipoproteins in the vessel wall activate the endothelial lining of the vessel, thereby attracting monocytes, which differentiate into macrophages upon migration through the endothelium. By taking up and storing the lipoproteins, these macrophages eventually become large foam cells and start secreting inflammatory mediators, cytokines and chemokines. The thereby created inflammatory environment attracts even more monocytes as well as other immune cells to the vessel wall, forming an atherosclerotic plaque[10].

In the atherosclerotic plaque, a wide variety of NF-κB inducers is present, ranging from modified lipoproteins to inflammatory mediators, free radicals and remnants of dead cells[11-13]. Therefore, activated NF-κB has been found in different cell types in the lesion, including macrophages, smooth muscle cells and endothelial cells[8,14]. To investigate the importance of this activation, we have previously studied models with either a

macrophage- [15] or endothelial cell-specific [16] ablation in NF-κB activation. While macrophage-specific deletion of IKK2 led to larger and more advanced lesions, endothelium-restricted NEMO deletion abrogated atherogenesis by impairing macrophage recruitment to the plaque.

In this paper we aimed at investigating the role of the NF-κB inhibitor IκBα in atherogenesis. Since full IκBα knockout mice die neonatal of hypergranulopoiesis and severe dermatitis[17-19], we used a conditional model with a myeloid specific deletion of IκBα[20]. Bone marrow from these LysMCre-*IκBα*$^{fl/fl}$ mice was transplanted into atherosclerosis-susceptible *ldlr*$^{-/-}$ mice to study the effect of myeloid IκBα deficiency on atherogenesis. We found that myeloid IκBα deficiency promotes atherogenesis by causing increased attraction of myeloid cells to the developing plaques without affecting other phenotypical characteristics of the lesions. Quite surprisingly, macrophage IκBα deficiency does not seem to affect the production of a number of NF-κB target genes *in vivo* nor *in vitro* but appears to be involved in the adhesion and recruitment of these cells to the atherosclerotic plaque.

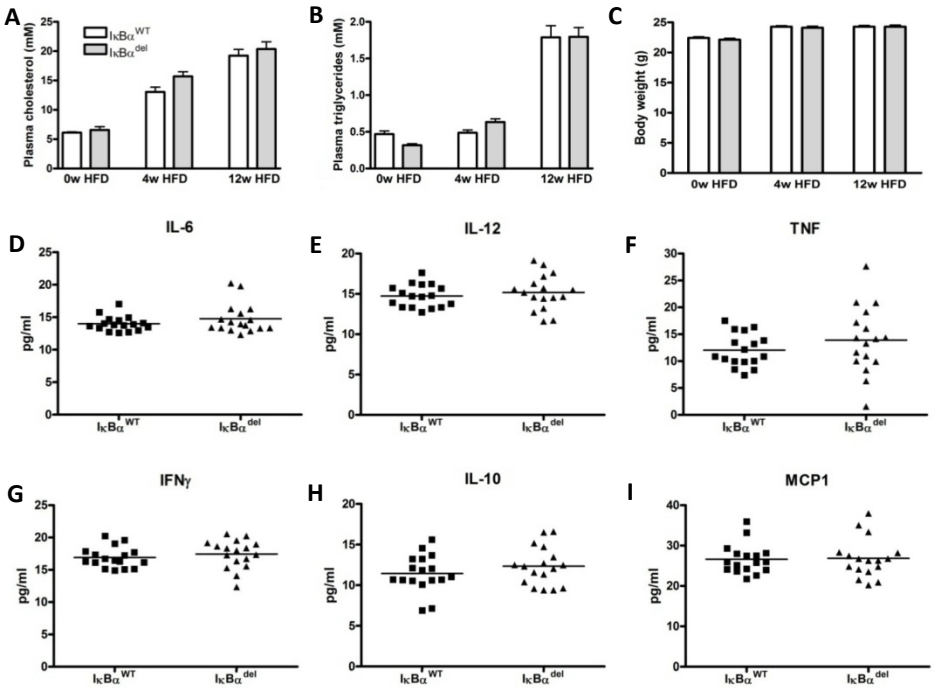

Figure 3.1: Plasma values and body weight of mice transplanted with either IκBα$^{fl/fl}$ or LysMCre-IκBα$^{fl/fl}$ bone marrow:

*(**A**) Cholesterol levels, (**B**) triglyceride levels and (**C**) body weight measured before the start of the high fat diet (0w HFD), after 4 weeks of diet (4w HFD) and upon sacrifice of the mice (12w HFD). (**D-I**) Plasma cytokine concentrations at the end of the experiment, measured by a bead array.*

Results

Myeloid IκBα deficiency promotes atherogenesis without altering plaque phenotype, body weight, plasma lipids and cytokines

To study the effect of myeloid IκBα deficiency on atherosclerosis, bone marrow was isolated from *IκBα*$^{fl/fl}$ and LysMCre-*IκBα*$^{fl/fl}$ mice, the latter having a myeloid-specific deletion of the *IκBα* gene[20]. This bone marrow was transplanted into irradiated *ldlr*$^{-/-}$ mice, resulting in atherosclerosis-susceptible mice that were either wildtype (IκBα^{WT}) or deleted (IκBα^{del}) for *IκBα* in their myeloid cells. Four weeks after transplantation, these mice were put on a high fat diet for 12 weeks in order to induce atherogenesis. The rise in plasma cholesterol, plasma triglycerides and body weight as a result of the diet was similar in both groups (Figures 3.1A, B and C). In addition, relative levels of circulating leukocyte populations (monocytes, granulocytes, T and B lymphocytes) after the transplantation did not differ between the groups (data not shown). After 12 weeks of diet, plasma levels of the NF-κB dependent pro-inflammatory cytokines IL-6, IL-12,

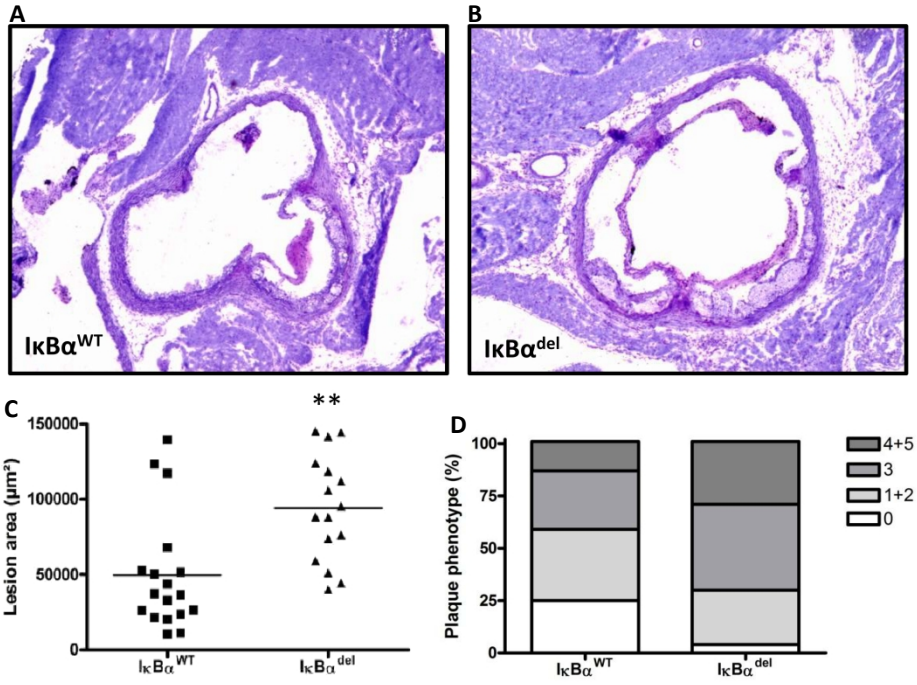

Figure 3.2: Myeloid IκBα deficiency promotes atherosclerosis in ldlr$^{-/-}$ mice:

*Representative pictures of toluidine blue-stained sections in the aortic root of (**A**) IκBα^{WT} or (**B**) myeloid-specific IκBα^{del} mice, original magnification x40. (**C**) Lesion area in the aortic root of IκBα^{WT} and IκBα^{del} mice (** p<0.01; n=18/16). (**D**) Lesion severity in the aortic root of IκBα^{WT} and IκBα^{del} mice (Chi square test; *** p<0.0001; n=54/48) was typed as absent (0), early (1+2), or advanced (4+5), as described before[15].*

TNF-α and IFNγ, the anti-inflammatory cytokine IL-10 and the chemokine MCP-1 (CCL2) were not influenced by the absence of myeloid IκBα (Figures 3.1D - I).

Upon sacrifice, atherosclerosis in the aortic root was analyzed. Interestingly, lesion area measurements using toluidine blue-stained sections (Figures 3.2A and B) showed a two-fold, significant increase in plaque formation in $I\kappa B\alpha^{del}$ mice compared to $I\kappa B\alpha^{WT}$ mice (49740 ± 9142 μm² vs. 94330 ± 8803 μm² for $I\kappa B\alpha^{WT}$ and myeloid-specific $I\kappa B\alpha^{del}$ respectively; p=0.0014) (Figure 3.2C). Moreover, classification of the lesions according to their severity showed that $I\kappa B\alpha^{del}$ mice had relatively more advanced and less early atherosclerotic lesions (Chi square test; p<0.0001) (Figure 3.2D).

With additional analyses on sections of the aortic root, the phenotype of these plaques was further characterized. Plaque-stabilizing collagen was stained with Sirius Red but no significant differences were found in collagen content between the two groups (Figure 3.3A). Also analysis of T cell and neutrophil content revealed no significant differences (Figures 3.3B and C). Finally, both groups appeared to have the same number of proliferating and apoptotic cells in their plaques, as shown by Ki-67 and a TUNEL stainings (Figures 3.3D and E). Thus despite having larger and more advanced lesions, the $I\kappa B\alpha^{del}$ lesions showed no other changes in their plaque characteristics.

Next to the lesions in the aortic root, atherosclerotic plaque formation was also assessed in the aortic arch. In RNA isolated from this tissue, a 49.6% increase in the expression of macrophage marker CD68 was found (p=0.0384, data not shown), indicating enhanced macrophage accumulation in the vessel wall of $I\kappa B\alpha^{del}$-transplanted mice.

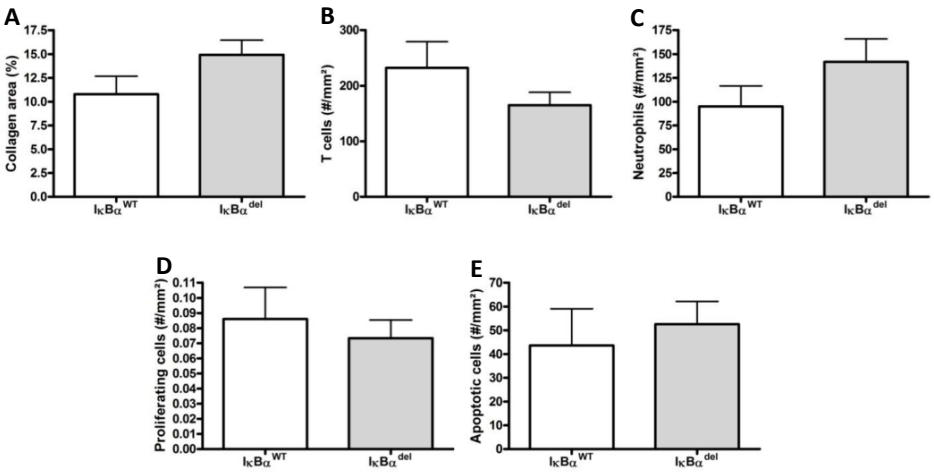

Figure 3.3: Myeloid IκBα deficiency does not influence plaque phenotypic characteristics:

*(**A**) Collagen content is not different between $I\kappa B\alpha^{WT}$ and $I\kappa B\alpha^{del}$ mice, as shown by a Sirius Red staining. Also the relative number of (**B**) T cells, (**C**) neutrophils, (**D**) proliferating and (**E**) apoptotic cells was similar in both groups.*

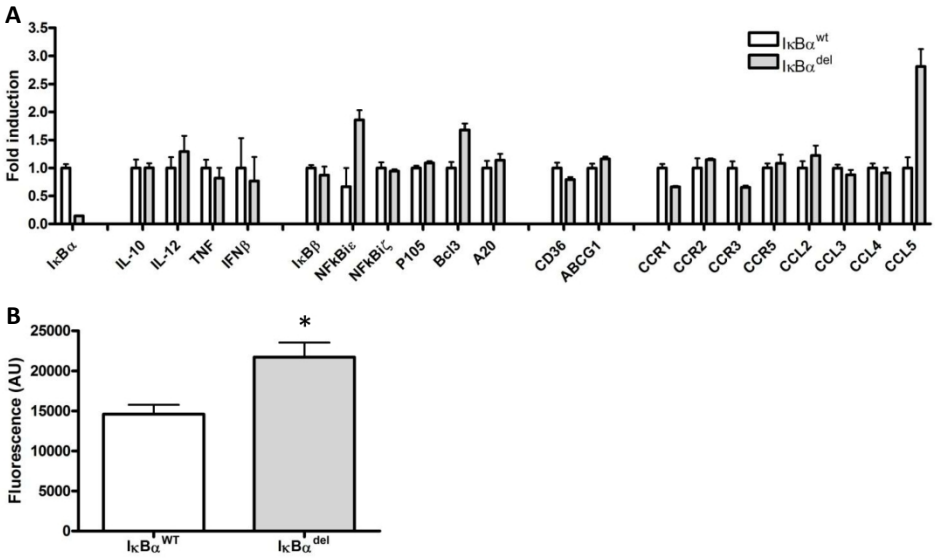

Figure 3.4: Deletion of IκBα in bone marrow-derived macrophages has limited effect on the expression of NF-κB-dependent and/or –regulating genes but enhances in vitro static macrophage adhesion:

*(**A**) Gene expression in triplicates of LysMCre-IκBα$^{fl/fl}$ macrophages was compared to IκBα$^{fl/fl}$ macrophages by Q-PCR. (**B**) Adhesion of fluorescently labeled macrophages to an endothelial monolayer (bars represent triplicate wells ± SEM; * p<0.05). Data shown are representative for at least 3 experiments.*

Myeloid IκBα deficiency promotes *in vitro* macrophage adhesion

To investigate the mechanism behind the enhanced atherogenesis in the IκBα^{del} mice, *in vitro* experiments were done. Surprisingly, the reduced production of IκBα shown in Figure 3.4A and Supplemental Figure S3.1A did not result in an increased activation of the p65 NF-κB subunit (Supplemental Figure S3.1B-C), suggesting a potent compensatory mechanism operating in the absence of IκBα. As a result, hardly any difference was found in the expression of some genes known to be NF-κB targets, NF-κB inhibitors or inflammatory mediators when assessed in unstimulated as well as LPS stimulated bone marrow-derived macrophages from LysMCre-*IκBα*$^{fl/fl}$ mice and compared to wild type cells (Figure 3.4A and Supplemental Figure S3.1D). However, the expression of the chemokine CCL5 (or RANTES) was significantly elevated 2.8 fold in macrophages lacking IκBα. CCL5 was previously shown to be involved in the adhesion of macrophages to endothelial cells. In line with these findings, static adhesion of IκBα^{del} macrophages to a monolayer of the bEND.5 endothelial cell line was significantly enhanced compared to wild-type macrophages (14610 ± 1163 AU vs. 21720 ± 1810 AU for IκBα^{WT} and IκBα^{del} respectively; p=0.0298) (Figure 3.4B).

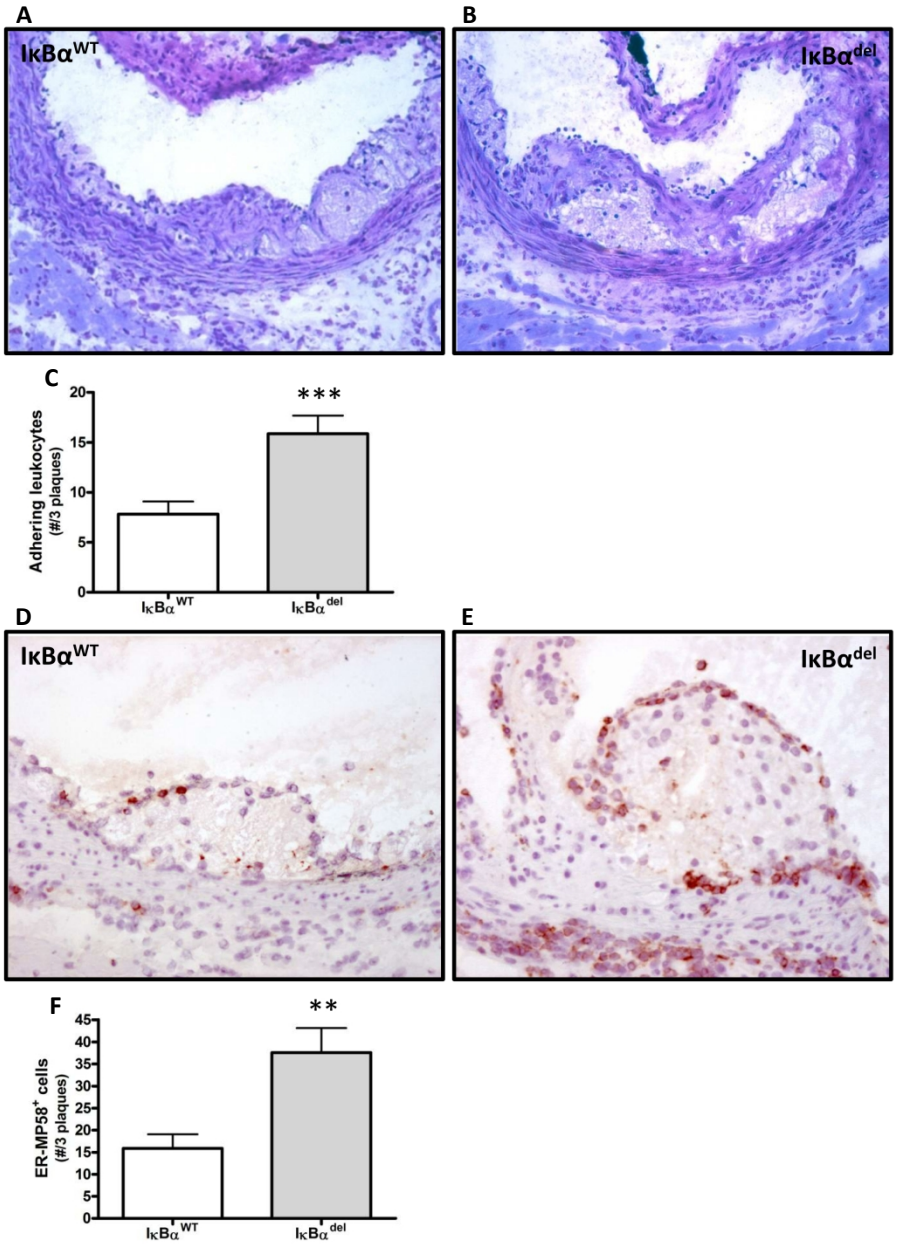

Figure 3.5: Increased leukocyte adhesion and newly recruited myeloid cells in IκBα^{del} mice:

*(**C**) In IκBα^{del} mice, more leukocytes adhere to the endothelial cell layer delineating the lesion (*** p<0.0001; n=17/17). Representative pictures from (**A**) IκBα^{WT} and (**B**) IκBα^{del} lesions, original magnification x200. (**F**) Lesions from IκBα^{del} mice contain more ER-MP58 positive cells, suggesting an increased attraction myeloid cells (** p<0.001; n=19/17). Representative pictures from (**D**) IκBα^{WT} and (**E**) IκBα^{del} lesions, original magnification x200.*

Atherosclerotic lesions in mice lacking myeloid IκBα show increased leukocyte adhesion and migration.

To investigate whether the observed increased *in vitro* macrophage adhesion could also be observed in the *in vivo* atherosclerosis model, cells adhering to luminal side of the endothelial cells covering the atherosclerotic plaques were quantified in the toluidine blue-stained sections. Indeed, the IκBα^{del} mice displayed a significantly higher number of adhering cells compared to the IκBα^{WT} mice (7.8 ± 1.3 cells vs. 15.9 ± 1.8 cells for IκBα^{WT} and IκBα^{del} respectively; p=0.0009) (Figures 3.5A - C), suggesting enhanced recruitment of new cells to the plaque in mice lacking IκBα in their myeloid cells.

To further analyze this recruitment of monocytes to the lesions, we used a marker that is specifically expressed on circulating immature myeloid cells but that is lost upon differentiation to macrophages. This marker, detected by the antibody ER-MP58, has previously been used in other studies to analyze recruitment of cells from the circulation to tissues[21-23]. Sections from the aortic root were stained with ER-MP58 and positive cells were quantified. As expected, these cells were predominantly observed on the luminal side of the plaque. However, also on the adventitial side, some myeloid recruitment could be observed. Interestingly, ER-MP58-positive cells were also seen in inflammatory regions in the vicinity of the plaques. Quantification showed that IκBα^{del} mice had significantly more newly recruited myeloid cells in their plaques compared to IκBα^{WT} mice (15.890 ± 3.164 cells vs. 37.590 ± 5.568 cells; p=0.0014) (Figures 3.5D - F). These data indicate that atherosclerotic lesions in the aorta of *ldlr*$^{-/-}$ mice lacking myeloid IκBα may be larger because of enhanced recruitment and infiltration of myeloid cells, suggesting that myeloid IκBα is important in regulating the migratory phenotype of cells in atherogenesis. A possible role for CCL5 in this mechanism is illustrated by the observation that the expression of this chemokine was significantly elevated in the aforementioned aortic arch-derived RNA (40.7% increase, p=0.0270, data not shown).

Discussion

In this paper we show that myeloid-specific deletion of the inhibitor of NF-κB, IκBα, in *ldlr*$^{-/-}$ mice results in larger atherosclerotic plaques without affecting the general plaque composition. Studying cell adhesion to the luminal side of the endothelial layer covering atherosclerotic plaques, we observed increased adhesion of leukocytes in the absence of myeloid IκBα. Through a marker specific for circulating myelomonocytic cells that is lost upon maturation to macrophages, ER-MP58, we found that lesions from mice lacking myeloid IκBα are characterized by more newly recruited leukocytes. Moreover, we found an increase in macrophage content in the vessel wall of the aorta.

These data suggest that IκBα in myeloid cells may have a pivotal role in regulating the recruitment of cells to atherosclerotic lesions.

Atherosclerosis is known to be driven by inflammation and aggravated by the production of pro-inflammatory cytokines[24]. Since cytokine expression is highly dependent on NF-κB, it can be expected that the atherosclerotic process is proportional to the activation level of this transcription factor. Indeed, Gareus et al. showed that endothelial-specific inhibition of NF-κB by NEMO deletion impaired macrophage recruitment to the plaque and hereby impaired atherogenesis[16]. Another paper, by Wolfrum et al., describes how haploinsufficiency for the NF-κB activation inhibitor A20 results in enhanced atherogenesis while A20 overexpression reduced plaque formation[25]. Our group also showed that the role of NF-κB in macrophages is not as straightforward. Myeloid-specific blocking of the canonical activation of NF-κB through deletion of IKK2 resulted in plaques that were not only larger but also more advanced and more necrotic, highlighting the fact that NF-κB also acts as an anti-apoptotic transcription factor and is involved in regulating anti-inflammatory mechanisms[15]. In contrast, the present study demonstrates that deletion of myeloid IκBα, aiming at myeloid-specific NF-κB activation, also induces larger and more advanced plaques but without affecting the plaque composition.

Studying gene expression in LysMCre-$I\kappa B\alpha^{fl/fl}$ bone marrow-derived macrophages and comparing it to wildtype cells, we found an increase in the expression of the chemokine RANTES (Released upon Activation, Normal T-cell Expressed and Secreted, or CCL5). This molecule belongs to a relatively limited set of chemokines and adhesion molecules that are described to be influencing atherogenesis through the recruitment of new cells to the plaque[26,27]. In a recent publication, our group showed that IFNβ-induced expression of CCL5 augments the static adhesion of macrophages to endothelial cells *in vitro* as well as the adhesion of leukocytes to the vessel wall at atherosclerosis-prone sites *in vivo*, thereby promoting the attraction of new cells to the plaques[28]. Macrophages from LysMCre-$I\kappa B\alpha^{fl/fl}$ mice indeed adhered more efficiently to an endothelial cell layer *in vitro*, while in the atherosclerosis model, more monocytes were found adhering to the endothelial layer covering the plaques in the $I\kappa B\alpha^{del}$ mice compared to the $I\kappa B\alpha^{WT}$ mice.

To confirm the hypothesis that the lesions in the $I\kappa B\alpha^{del}$ mice were larger because of an increased attraction of leukocytes, we applied a staining detecting a marker specific for immature myeloid cells, ER-MP58. The target is an antigen with a yet unknown function, which is present on the bone marrow-derived, myeloid-committed progenitor cells. It continues to be expressed on neutrophils and monocytes but disappears progressively upon maturation of the M-CSF-responsive cells to macrophages[21,29]. Thus, both Ly-6C-high and Ly-6C-low subsets of circulating monocytes are positive for ER-MP58 while tissue macrophages have lost the marker[29,30]. Previously, detection of this marker has been used to distinguish infiltrating immature myeloid cells from the

resident macrophages already present within the site of inflammation, both in thioglycollate elicited macrophage recruitment to the peritoneum[21,22] and in the restoration of the Kupffer cell population in the liver, following injection with liposome-entrapped dichloromethylene diphosphonate[23]. In this paper, we show that ER-MP58 is also a valid marker for newly recruited myeloid cells to the atherosclerotic plaque, with a positivity that is limited to small, recently infiltrated cells in the vicinity of the luminal plaque surface and inflammatory regions.

Contrary to the upregulation of CCL5 expression in the LysMCre-*IκBα*$^{fl/fl}$ macrophages, many typical NF-κB-dependent genes were not influenced by the deletion of IκBα. This suggests that in these cells, NF-κB is not continuously activated, but inhibited by other feedback mechanisms which, like IκBα, prevent NF-κB translocation to the nucleus or terminate NF-κB activation by exporting it back to the cytoplasm. Indeed, studying the nuclear translocation of several NF-κB subunits, we found that the LysMCre-*IκBα*$^{fl/fl}$ macrophages had the same degree of translocation as wildtype cells (data not shown) and no increase in p65 phosphorylation was observed in the IκBα^{del} macrophages (Supplemental Figure S3.1B-C). In addition, only mild to no upregulation of other IκB family members was detected by gene expression analysis, indicating alternative mechanisms of regulation of NF-κB dependent transcription. Recent studies indeed show that, besides IκB inhibitors, also nuclear ubiquitin ligases can terminate chronic NF-κB activation by its nuclear degradation, a mechanism that might compensate the deletion of IκBα[31].

In conclusion, we found that myeloid-specific deletion of IκBα resulted in an upregulation of the chemokine CCL5 and enhanced static *in vitro* adhesion of macrophages to an endothelial cell layer. Moreover, this correlated *in vivo* with increased leukocyte adhesion to the activated endothelial lining of the blood vessel, enhanced recruitment of ER-MP58^{+} immature myeloid cells to the atherosclerotic plaque and increased atherosclerotic lesion formation in the aortic root and arch. Hereby we show that the role of myeloid IκBα in the regulation of inflammation is complex but that it is involved in the recruitment of macrophages to the atherosclerotic plaque.

Materials and methods

Mice

C57BL/6 mice and *ldlr*$^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME). *IκBα*$^{fl/fl}$ mice on a C57BL/6 background were described before[32]. All animal experiments were approved by the DierExperimenten Commissie (DEC) of the Maastricht University (permit numbers 2005-090 and 2009-168).

Bone marrow transplantation

One week before transplantation, female *ldlr*$^{-/-}$ mice were housed in filter top cages and provided with acidified water containing neomycin (100mg/l; Gibco, Breda, The Netherlands) and polymyxin B sulphate (6x10^4 U/l; Gibco). The animals received 10Gy total body irradiation and on the following day, bone marrow was isolated from 6 LysMCre-IκBα$^{fl/fl}$ mice (IκBα^{del}) and 6 IκBα$^{fl/fl}$ littermates (IκBα^{wt}) and 10^7 cells/mouse were injected intravenously to rescue the hematopoietic system of the irradiated mice. Four weeks after the transplantation, mice were fed a high fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 12 weeks.

Mouse blood parameters

At several time points during the *in vivo* atherosclerosis experiment, blood was drawn from the mice. Plasma lipid levels were monitored enzymatically (Sigma Aldrich, Zwijndrecht, the Netherlands) and plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array kit (BD-Pharmingen, San Diego, CA). Leukocytes were counted using a Coulter counter and blood cell distribution was quantified by flow cytometry after antibody staining with either Mac1-PE and Gr1-FITC for macrophages and granulocytes or 6B2-PE and KT3-FITC for B- and T-cells (BD-Pharmingen, Erembodegem, Belgium).

Atherosclerosis analysis

Upon sacrifice, the hearts from the bone marrow transplanted mice were taken out and cut perpendicular to the heart axis just below the atrial tips. Tissue was frozen in tissue-tec (Shandon, Veldhoven, The Netherlands) and cut into sections of 7 µm as described before[15]. Serial cross-sections from every 42 µm were stained with toluidine blue. All lesion areas were quantified using Adobe Photoshop software. The lesions were also typed according to severity as early, moderate and advanced, as described before[15].

Immunohistochemical staining

Lesions from the aortic root were fixed in acetone and incubated with antibodies against neutrophils (1A8, BD-Pharmingen), T cells (KT3, directed against CD3, a gift from G. Kraal), proliferating cells (Ki-67, Dako, Glostrup, Denmark) and newly recruited macrophages (ER-MP58, P. Leenen), followed by detection with a biotin labeled rabbit anti-rat antibody and staining with the ABC kit (Vector Labs, Burlingame, CA). Apoptotic cells in the plaques were stained by the TUNEL staining (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Collagen areas were analyzed on Sirius red stained sections. Adhering leukocytes were quantified on toluidine blue-stained sections.

In vitro murine bone marrow macrophage culture

Bone marrow cells were isolated from femurs and tibiae of either wild-type (IκBα$^{fl/fl}$) or deleted (LysMCre-IκBα$^{fl/fl}$) mice. Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, The Netherlands) with 10% heat-inactivated fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 ug/ml), and L-glutamine 2mM (all GIBCO Invitrogen, Breda, The Netherlands) supplemented with 15% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages (BMM), as described previously[15].

Western blotting

Protein was isolated from BMM with an SDS lysis buffer, supplemented with complete protease inhibitor cocktail (Roche Diagnostics) and PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics). After Western blotting, blots were incubated with P-p65 antibody (1:500, Cell Signaling, Danvers, MA) in PBS with 0.05% Tween and 5% BSA (Sigma Aldrich).

Gene expression

RNA was isolated from BMM with the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) or from snap-frozen aortic arches with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). 500 ng total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative PCR (Q-PCR) was performed using 10 ng cDNA, 300 nM of each primer, and SensiMix (Quantace-Bioline, London, UK) in a total volume of 20 µl. All gene expression levels were corrected for cyclophilin A as housekeeping gene. Primer sequences are available upon request.

In vitro adhesion assay

A confluent monolayer of bEND5 endothelial cells was grown in fluorescence 96-well microplates (Greiner Bio-one, Frickenhausen, Germany). Triplicate wells were incubated for 30 min with 10^5 BMM that had been fluorescently labeled with a PKH67 dye according to the manufacturer's instructions (Sigma Aldrich, Zwijndrecht, The Netherlands). Subsequently, the wells were washed three times with the aforementioned macrophage medium, and adherent cells were measured by fluorometry in a Synergy HT microtiter plate reader (BioTek, Bad Friedrichshall, Germany) at an excitation of 485 nm and an emission of 520 nm.

Statistical analysis

The statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between 2 groups were evaluated using a t-test, unless stated otherwise.

Values are represented as mean ± SEM. A *P* value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

References

1. Stewart, C.R., *et al.* CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol* 11, 155-161 (2010).
2. Wiesner, P., *et al.* Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor kappab and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. *Circ Res* 107, 56-65 (2010).
3. Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18, 6853-6866 (1999).
4. Tergaonkar, V., Correa, R.G., Ikawa, M. & Verma, I.M. Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. *Nature cell biology* 7, 921-923 (2005).
5. Karin, M. & Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annual review of immunology* 18, 621-663 (2000).
6. Vallabhapurapu, S. & Karin, M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annual review of immunology* 27, 693-733 (2009).
7. de Winther, M.P., Kanters, E., Kraal, G. & Hofker, M.H. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* 25, 904-914 (2005).
8. Hajra, L., *et al.* The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proceedings of the National Academy of Sciences of the United States of America* 97, 9052-9057 (2000).
9. Ross, R. Atherosclerosis is an inflammatory disease. *Am Heart J* 138, S419-420 (1999).
10. Lusis, A.J. Atherosclerosis. *Nature* 407, 233-241 (2000).
11. Brand, K., *et al.* Dysregulation of monocytic nuclear factor-kappa B by oxidized low-density lipoprotein. *Arterioscler Thromb Vasc Biol* 17, 1901-1909 (1997).
12. Monaco, C., *et al.* Canonical pathway of nuclear factor kappa B activation selectively regulates proinflammatory and prothrombotic responses in human atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America* 101, 5634-5639 (2004).
13. Xanthoulea, S., Curfs, D.M., Hofker, M.H. & de Winther, M.P. Nuclear factor kappa B signaling in macrophage function and atherogenesis. *Curr Opin Lipidol* 16, 536-542 (2005).
14. Brand, K., *et al.* Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *The Journal of clinical investigation* 97, 1715-1722 (1996).
15. Kanters, E., *et al.* Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *The Journal of clinical investigation* 112, 1176-1185 (2003).
16. Gareus, R., *et al.* Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. *Cell metabolism* 8, 372-383 (2008).
17. Beg, A.A., Sha, W.C., Bronson, R.T. & Baltimore, D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev* 9, 2736-2746 (1995).
18. Gerondakis, S., *et al.* Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models. *Oncogene* 25, 6781-6799 (2006).
19. Klement, J.F., *et al.* IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. *Mol Cell Biol* 16, 2341-2349 (1996).

20. Rupec, R.A.*, et al.* Stroma-mediated dysregulation of myelopoiesis in mice lacking I kappa B alpha. *Immunity* 22, 479-491 (2005).
21. Chan, J., Leenen, P.J., Bertoncello, I., Nishikawa, S.I. & Hamilton, J.A. Macrophage lineage cells in inflammation: characterization by colony-stimulating factor-1 (CSF-1) receptor (c-Fms), ER-MP58, and ER-MP20 (Ly-6C) expression. *Blood* 92, 1423-1431 (1998).
22. Cook, A.D., Braine, E.L. & Hamilton, J.A. The phenotype of inflammatory macrophages is stimulus dependent: implications for the nature of the inflammatory response. *J Immunol* 171, 4816-4823 (2003).
23. Yamamoto, T.*, et al.* Repopulation of murine Kupffer cells after intravenous administration of liposome-encapsulated dichloromethylene diphosphonate. *Am J Pathol* 149, 1271-1286 (1996).
24. Tedgui, A. & Mallat, Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 86, 515-581 (2006).
25. Wolfrum, S., Teupser, D., Tan, M., Chen, K.Y. & Breslow, J.L. The protective effect of A20 on atherosclerosis in apolipoprotein E-deficient mice is associated with reduced expression of NF-kappaB target genes. *Proceedings of the National Academy of Sciences of the United States of America* 104, 18601-18606 (2007).
26. Zernecke, A. & Weber, C. Chemokines in the vascular inflammatory response of atherosclerosis. *Cardiovasc Res* 86, 192-201.
27. Zernecke, A., Shagdarsuren, E. & Weber, C. Chemokines in atherosclerosis: an update. *Arterioscler Thromb Vasc Biol* 28, 1897-1908 (2008).
28. Goossens, P.*, et al.* Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. *Cell metabolism* 12, 142-153 (2010).
29. Leenen, P.J., de Bruijn, M.F., Voerman, J.S., Campbell, P.A. & van Ewijk, W. Markers of mouse macrophage development detected by monoclonal antibodies. *J Immunol Methods* 174, 5-19 (1994).
30. Sunderkotter, C.*, et al.* Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* 172, 4410-4417 (2004).
31. Natoli, G. & Chiocca, S. Nuclear ubiquitin ligases, NF-kappaB degradation, and the control of inflammation. *Sci Signal* 1, pe1 (2008).
32. Rebholz, B.*, et al.* Crosstalk between keratinocytes and adaptive immune cells in an IkappaBalpha protein-mediated inflammatory disease of the skin. *Immunity* 27, 296-307 (2007).

Chapter 4

A survivin-targeted lentiviral vaccination strategy reduces murine atherogenesis

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Submitted

Abstract

Survivin is an inhibitor of apoptosis and is highly expressed in virtually all tumors but has low to undetectable levels in healthy cells. Therefore, survivin has been a target in cancer therapies, including immunotherapy against survivin-positive cells. Recently, survivin expression was observed in macrophages that infiltrate early human atherosclerotic lesions. In this paper, we confirmed the presence of survivin-positive cells in murine atherosclerotic plaques. Subsequently, we targeted atherosclerosis by inducing a cytotoxic T cell response against survivin-positive plaque macrophages. We immunized atherosclerosis-susceptible mice using a lentivirus-based vaccination strategy, which indeed reduced atherosclerotic lesion size and promoted the migration of $CD3^+$ T cells and $CD8^+$ cytotoxic T cells into the plaque. These experiments are the first to show the therapeutic potential of immunization against plaque-infiltrating macrophages and thereby offer a novel perspective for immunotherapy-based strategies in combating atherosclerosis.

Introduction

Survivin (also known as baculoviral inhibitor of apoptosis repeat-containing 5, BIRC5) is a member of the Inhibitor of Apoptosis (IAP) family of proteins[1] and is predominantly studied as a tumor-associated protein. While its expression in healthy cells is usually low to undetectable[2] and restricted to the G2/M phase of mitosis with a rapid downregulation in the G1 phase, virtually all tumors exhibit an excessive and cell cycle-independent expression, making survivin the fourth most upregulated gene in tumor transcriptomes[3].

Survivin regulates two essential cellular processes that both contribute to tumor growth. As an IAP family member, survivin inhibits apoptosis. Indeed, its overexpression attenuates cell death, while inhibiting its function results in spontaneous cell death and a higher sensitivity to apoptotic stimuli[4]. It is, however, not a direct caspase inhibitor[5], and the mechanism behind its effect on apoptosis is still unclear. In addition to blocking apoptosis, survivin also regulates mitosis through its effects on multiple spindle microtubule functions and mitotic checkpoints, promoting proliferation by facilitating accurate sister chromatid segregation and stabilization of microtubules in late mitosis[6,7]. Because cell cycle progression and resistance to cell death are two factors that are essential to tumor formation, survivin upregulation may be a key step in the initiation of tumorigenesis[8]. These properties make survivin an attractive universal target for anti-tumor drugs and therapies[4]. Several strategies, including small-molecule antagonists, dominant-negative mutants, small interfering RNA (siRNA), ribozymes, anti-sense oligonucleotides, immunotherapy and survivin promoter-induced TRAIL expression, have been tested in experimental models, and some strategies have advanced to human phase I or II clinical trials, all without significant cross-reactivity-related side effects[9-12].

Interestingly, Blanc-Brude et al. described survivin to also be detectable in human atherosclerotic plaques[13]. Atherosclerosis is a chronic inflammatory disease of the vascular wall that gradually causes a variety of acute, life-threatening outcomes, such as myocardial infarction and stroke. It is initiated when low-density lipoproteins (LDL) accumulate in the subendothelial space and are chemically modified. This mobilizes circulating monocytes to the vessel wall to clear the modified LDL. The accumulation of these cells forms an early atherosclerotic plaque or fatty streak. Subsequently, the plaque evolves to a more advanced state by the attraction of more blood cells, the growth of a fibrous cap and the appearance of a necrotic core, a region in the plaque where the debris of inefficiently cleared apoptotic and necrotic cells gathers. The susceptibility of the plaque to rupture and cause acute symptoms is determined by the balance between stabilizing (e.g., cap thickness) and destabilizing (e.g., necrotic core size) factors.

In the aforementioned study, macrophage colony-stimulating factor (M-CSF) is able to upregulate survivin expression in macrophages *in vitro*[13]. M-CSF is abundantly present in atherosclerotic plaques where it promotes macrophage attraction and survival[14]. Prolonged *in vitro* exposure to oxidized LDL (oxLDL), however, abolishes survivin expression[13]. Indeed, immunohistochemical analysis of human atherosclerotic plaques revealed that $CD68^+$ macrophages are positive for survivin at the luminal side of early fatty streaks, while in more advanced plaques, survivin-positive cells were rarely found[13]. This suggests that newly infiltrated macrophages in early atherosclerotic plaques express survivin under the influence of M-CSF, possibly as a protective mechanism against apoptosis provoked by radical oxygen species and lipid degradation products within the lesion[15]. Once the macrophages become foam cells, however, they lose their survivin expression. Thereby they become vulnerable to apoptosis, contributing to necrotic core formation.

In this paper, we identify survivin as a new specific immune target in atherosclerosis by showing that survivin-expressing cells are detectable in murine plaques. Analogous to cancer models, in which cellular immunity against survivin-expressing cells selectively kills tumor cells without significant side effects[16,17], we applied a lentiviral vaccination strategy to induce cellular immunity against newly infiltrating macrophages in the plaques. We show that such survivin-based immunotherapy effectively reduced atherosclerotic plaque size and was accompanied by an enhanced recruitment of $CD3^+$ T cells and $CD8^+$ cytotoxic T cells to the lesions.

RESULTS

Survivin is detected in murine atherosclerotic plaques and macrophages

Cryosections of atherosclerotic lesions from $LDLR^{-/-}$ mice fed a high-fat diet for 12 weeks were immunohistochemically stained for survivin. In the plaques, survivin-positive cells were clearly detectable (Figure 1 A-B). Morphologically, these cells were identified as macrophages, and they were located predominantly on the luminal side of the plaque, similar to the survivin-positive cells found in human fatty streaks [13]. No staining was observed in the media or in the adjacent aorta, indicating that survivin expression was specific to the plaque-infiltrating macrophages, in addition to its usually assumed restriction to tumor cells, apoptosis-related fetal tissues[18], sites of vascular injury[19] and a selection of self-renewing cells[2].

Analysis of microarray data generated by our group[20] showed that treating murine bone marrow-derived macrophages *in vitro* with oxLDL, but not with acetylated LDL (acLDL) or unmodified LDL, reduced *survivin* expression by 48% ($p=0.0068$; Figure 1 C).

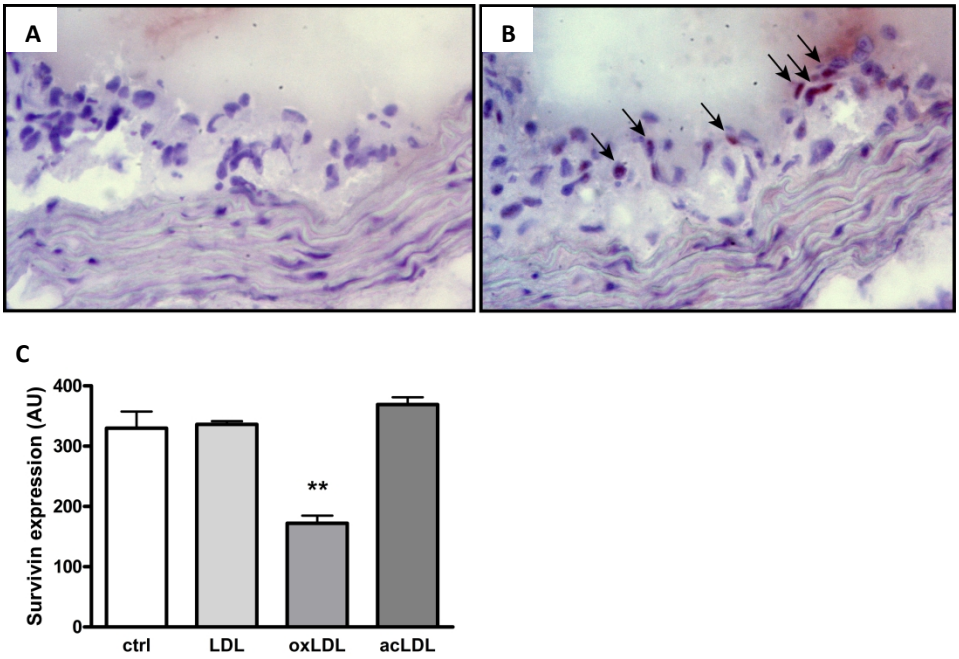

Figure 4.1: Survivin expression in murine atherosclerotic plaques and macrophages:

***A-B**: Murine atherosclerotic plaques were immunohistochemically stained without (A) or with (B) a survivin-detecting antibody (original magnification x200). The survivin-expressing cells are indicated by arrows. **C**: Survivin gene expression in murine bone marrow-derived macrophages incubated for 24h with non-modified LDL, oxidized LDL or acetylated LDL.*

Validation of a survivin-specific cytotoxic T cell response upon lentiviral vaccination

Inhibition of survivin expression and elimination of survivin-positive cells have been successfully applied as therapies in murine tumor models and cancer patients[10]. To evaluate whether survivin-positive cells in atherosclerotic plaques can be targeted in a similar way, we developed a lentiviral vector-based vaccination strategy aimed to skew the immune system toward autoreactivity against survivin-presenting cells. The murine *survivin* gene was cloned into a lentiviral vector containing a CMV promoter. Moreover, this vector encodes the non-functional truncated nerve growth factor receptor (tNGFR) as a marker gene[21]. Viruses generated from this vector ('SURV') or from the empty control vector ('tNGFR') were subsequently used for vaccination.

To verify the ability of these viruses to induce a murine survivin-specific immune reaction, BALB/c mice were injected in the footpad with one of the viral vectors. Four days after immunization, splenocytes from the mice were isolated and re-stimulated *in vitro* with the survivin-expressing BALB/c-derived B cell lymphoma cell line A20. Subsequently, the induction of cytotoxic T cells was evaluated in a ^{51}Cr-release assay. In this experiment, the splenocytes were co-cultured with radioactively labeled A20 cells

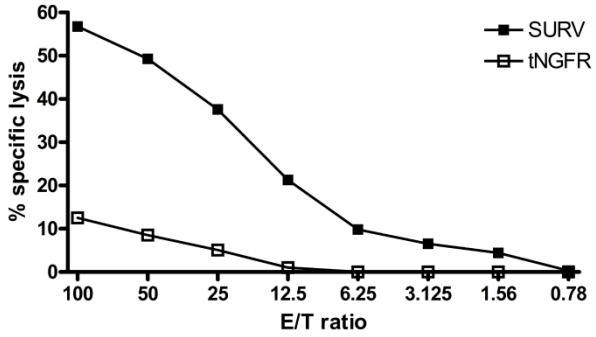

Figure 4.2: **In vitro *cytotoxicity assay:***

Splenocytes from mice that were vaccinated with either control (tNGFR) or survivin (SURV) lentiviruses were re-stimulated with survivin-expressing cells, after which they were co-cultured with ^{51}Cr-labeled A20 cells at different E/T ratios, and lysis was analyzed.

at different effector/target (E/T) ratios, and specific cell lysis was analyzed. The results show that vaccination with a survivin-encoding lentivirus significantly induced cytolytic activity against A20 cells (two-way ANOVA, p=0.0141; Figure 2).

LDLR$^{-/-}$ vaccination and general pathology

To assess the effect of a survivin-targeted vaccination on atherosclerosis development, 9-week-old LDLR$^{-/-}$ mice were injected with either 'SURV' (n=16) or 'tNGFR' (n=15) lentiviruses. They received additional boost injections after 1 and 4 weeks. One week after the first injection, the mice were placed on a high-fat diet to induce atherogenesis, which was maintained for 11 weeks (Supplemental Figure S4.1). During this period, both groups of mice gained equally in total body weight (Supplemental Figure S4.2A), and both groups had similarly raised plasma cholesterol and triglyceride levels (Supplemental Figure S4.2B-C). No mice died or showed any sign of discomfort during the experiment. Upon sacrifice, no macroscopic signs of pathology were visible in either of the vaccinated groups, and the microscopic examination of the small intestine, kidneys, liver and skin did not show any indication of tissue damage or immune cell aggregation. The plasma values and pathology of the lentivirus-injected groups did not differ from that of a group of PBS-injected control mice (n=15).

Atherosclerotic plaque analysis

After 11 weeks on a high-fat diet, the mice were sacrificed, and cryosections of the aortic root were obtained to analyze the effect of vaccination on atherosclerosis. Compared with the 'tNGFR' group, 'SURV'-vaccinated mice had plaques that were 31% smaller (124,900 ± 6,472 µm² vs. 85,950 ± 10,250 µm², respectively, p=0.0080; Figure 3 A-B). We observed no effect from the lentiviral treatment itself; lesions of the 'tNGFR'-vaccinated mice were equal in size compared with PBS-injected mice (data not shown).

Both groups of vaccinated mice had equal percentages of plaque-stabilizing collagen in their lesions (7.002 ± 1.050% for 'tNGFR' vs. 6.340 ± 1.050% for 'SURV', p=0.6647; Supplemental Figure S4.2D), while the necrotic core area was remarkably yet insignificantly increased upon 'SURV' treatment (3.222 ± 0.6864% for 'tNGFR' vs. 5.705 ± 1.181% for 'SURV', p=0.0834; Supplemental Figure S4.2E).

Because the lentiviral vaccination was designed to induce a cytotoxic response against survivin-expressing cells, the number of T cells within the plaque was assessed using CD3 staining. We observed an increase in $CD3^+$ T cells in the plaques of 'SURV'-vaccinated mice (113.2 ± 21.65 T cells/mm² for 'tNGFR' vs. 611.7 ± 92.19 T cells/mm² for 'SURV', p=0.0001; Figure 3 C-D). Focusing further on cytotoxic T cells, we also found a higher lesion content of $CD8^+$ cells in 'SURV'-vaccinated mice (37.64 ± 8.472 $CD8^+$ cells/mm² for 'tNGFR' vs. 84.68 ± 18.06 $CD8^+$ cells/mm² for 'SURV', p=0.0306; Figure 3 E-F), suggesting the recruitment of survivin-targeted cytotoxic T cells.

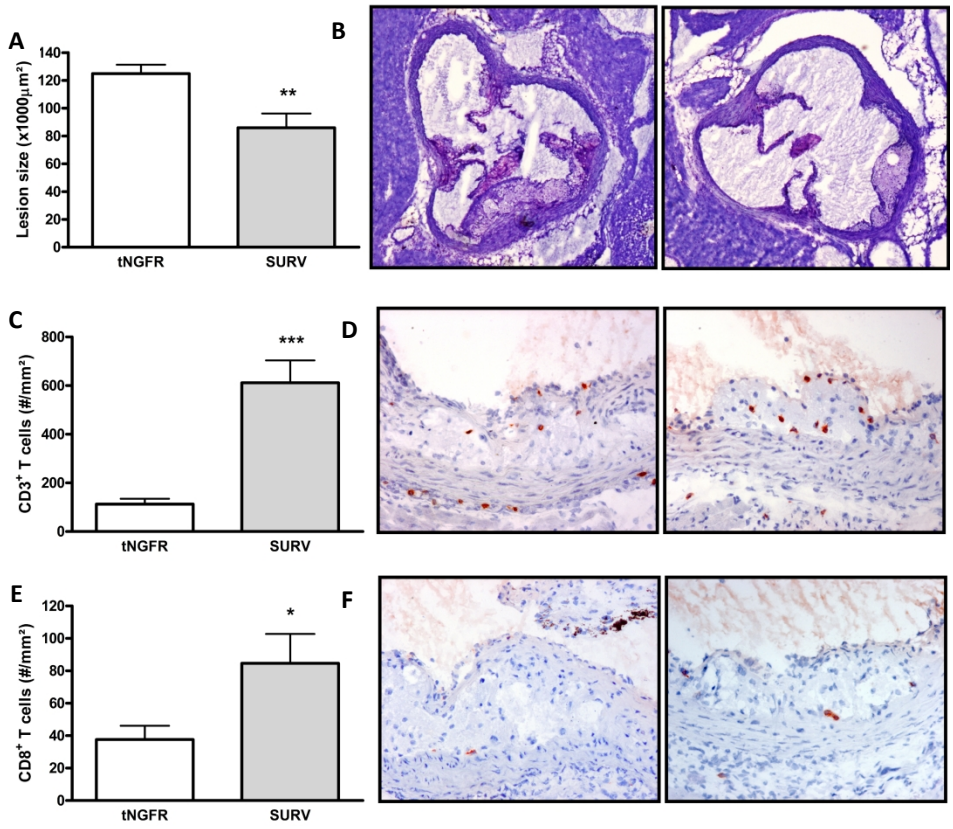

Figure 4.3: Atherosclerotic plaque size and phenotype:

***A-B**: Sections of the aortic root of mice treated with either control (tNGFR) (n=15) or survivin (SURV) (n=16) lentivirus were stained with toluidine blue to quantify the lesion size. **C-D**: The numbers of $CD3^+$ T cells in the plaques of tNGFR or SURV treated mice with representative pictures. **E-F**: The numbers of $CD8^+$ cytotoxic T cells in plaques of tNGFR or SURV treated mice with representative pictures.*

DISCUSSION

In chronic diseases where leukocyte recruitment to the site of inflammation has a detrimental effect on outcome[22], knowledge of specific markers for the infiltrating cells can be very useful for both analysis and treatment purposes. While no survivin expression is present in healthy blood vessels[18,19], the presence of survivin-positive cells has been demonstrated in human atherosclerotic lesions[13]. In this paper, we evaluated murine lesions for the presence of cells that express survivin and subsequently targeted them with a lentiviral vaccination strategy.

Blanc-Brude et al. described how M-CSF induces the expression of survivin in macrophages entering the human plaque. However, these survivin-positive cells were absent in the adventitial region as well as in more advanced plaques, which was attributed to a downregulation of the expression evoked by long-term exposure to oxidized lipids within the plaque[13]. We confirmed the presence of survivin-positive cells in a confined region within murine plaques and identified them morphologically as newly recruited macrophages. Furthermore, we demonstrated *in vitro* that murine bone-marrow-derived macrophages cultured in the presence of M-CSF express survivin and lose this expression upon treatment with oxLDL, but not with acLDL or unmodified LDL. This indicates that the survivin downregulation in foam cells is specifically related to the oxidative modification of lipoproteins rather than the intracellular accumulation of cholesteryl esters. Taken together, these data indicate that survivin is a specific marker for newly recruited macrophages in murine atherosclerotic plaques that is lost upon foam cell formation.

The specificity of this marker allows targeting of these cells through the induction of a cytotoxic immunity. A lentiviral vaccination strategy was designed in analogy to tumor cell-targeting vaccines, where it was shown previously that direct administration of similar lentiviral vectors to mice results in the induction of transgene-specific T cell responses[23,24]. In order to evaluate the potency of the lentiviral injections to induce cytotoxicity against survivin-expressing cells, we measured the level of cell death in a survivin-positive cell line co-cultured with splenocytes from vaccinated mice. The observed induction in cytotoxicity is comparable to results from earlier immunization studies using the same constructs with a different insert[24].

Until now, therapeutic options in atherosclerosis have been mainly restricted to plasma lipid lowering, either through dietary changes or pharmaceutical intervention. Recently, experimental immune modulation gained attention and has begun to be used in the clinic. To achieve a more targeted effect than systemic immune suppression, however, an immune response against atherosclerosis-specific antigens is needed. Active or passive immunization with oxLDL or with specific epitopes derived from oxidized phospholipids or apoB-100 has been shown to reduce plaque size[25,26]. Other immune therapies that successfully attenuated experimental atherosclerosis include promoting

regulatory T cells through oral HSP60 tolerance induction[27], skewing the helper T cells towards a regulatory T cell or Th2 response[28] and inducing cellular immunity against CD99[29] or vascular endothelial growth factor receptor 2 (VEGFR2)[30].

Analysis of the atherosclerosis in LDLR$^{-/-}$ mice revealed that vaccination with the 'SURV' lentiviruses resulted in significantly smaller lesions containing more CD3^{+} T cells and CD8^{+} cytotoxic T cells, without affecting other tissues or the mice' health. Moreover, their lesions displayed a trend towards an increase in necrotic core area, which may be a reflection of the lysis of survivin-expressing cells within the plaques and may suggest that 'SURV' vaccination provokes plaque instability over time, an important issue that should be further investigated in the future.

In conclusion, the lentiviral vaccination strategy applied in this murine atherosclerosis model reduced the lesion size and induced the presence of intra-plaque T cells and cytotoxic T cells. This confirms the feasibility of this novel approach, which specifically targets cells in the atherosclerotic plaque with a survivin-specific immune response without significant side effects. This approach is analogous to existing cancer therapies that target survivin-expressing tumor cells. Future studies should further investigate the long-term effects of lentiviral vaccination on plaque size and composition and also study whether therapeutic and prophylactic vaccinations have similar outcomes.

METHODS

Mice

BALB/c mice and *ldlr*$^{-/-}$ mice on a C57BL/6 background were obtained from Harlan (Horst, The Netherlands) and Jackson Laboratory (Bar Harbor, ME), respectively. All animal experiments were approved by the Leiden University and the Vrije Universiteit Brussel.

Survivin expression

Macrophage *survivin* expression was assessed by Affymetrix microarray, as described before[20].

Lentivirus production

The murine *survivin* gene (a kind gift from Dr. E.M. Conway, Katholieke Universiteit Leuven, Belgium), was cloned into the pSINT vector, which was previously described [21]. This transfer plasmid ('SURV') or the empty vector ('tNGFR') was co-transfected into the human embryonal kidney (HEK) 293T cell line together with the envelope (pMD.g) and packaging (pCMVΔR8.9) plasmids (a kind gift from Dr. Trono, University of Geneva, Switzerland), using the FuGENE® HD transfection reagent (Roche, Mannheim, Germany). Viruses were harvested from the supernatant on three consecutive days and

concentrated by ultracentrifugation, as previously described[21]. Virus titers were determined using the NucleoSpin RNA Virus kit (Macherey-Nagel, Düren, Germany) and the Lenti-X qRT-PCR Titration kit (Clontech, Mountain View, CA).

Mouse immunization and cytotoxicity assay

BALB/c mice were immunized by injection of 10^7 transducing units (TU) of the 'SURV' or 'tNGFR' lentivirus in the footpad. The induction of survivin-specific T cells was analyzed by isolation of spleen cells and re-stimulation for 5 days by co-culture with mitomycin C-treated A20 cells at a responder/stimulator ratio of 1:2. Subsequently, splenocytes were co-cultured for 4 hours with ^{51}Cr-labeled A20 cells at different effector/target (E/T) ratios. Cell lysis was measured by ^{51}Cr-release in the supernatant with a β counter as previously described[21,24].

Atherosclerosis analysis

Ldlr$^{-/-}$ mice were immunized by injection of 10^7 TU of 'SURV' (n=16) or 'tNGFR' lentivirus (n=15) in the footpad. The effect of the virus injection was assessed by injecting a parallel group with PBS (n=15). The mice were fed a high fat diet (15% cocoa butter, 0.25% cholesterol) for 11 weeks and subsequently sacrificed for analysis as described previously[22]. Survivin staining was performed using a rabbit polyclonal antibody (Novus Biologicals, Littleton, CO).

Statistical analysis

Statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between 2 groups were evaluated using a t-test, unless stated otherwise. Values are represented as mean ± SEM. A *P* value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

REFERENCES

1. Ambrosini, G., Adida, C. & Altieri, D.C. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* **3**, 917-921 (1997).
2. Fukuda, S. & Pelus, L.M. Survivin, a cancer target with an emerging role in normal adult tissues. *Mol Cancer Ther* **5**, 1087-1098 (2006).
3. Velculescu, V.E.*, et al.* Analysis of human transcriptomes. *Nat Genet* **23**, 387-388 (1999).
4. Altieri, D.C. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer* **3**, 46-54 (2003).
5. Banks, D.P.*, et al.* Survivin does not inhibit caspase-3 activity. *Blood* **96**, 4002-4003 (2000).
6. Yang, D., Welm, A. & Bishop, J.M. Cell division and cell survival in the absence of survivin. *Proc Natl Acad Sci U S A* **101**, 15100-15105 (2004).
7. Li, F.*, et al.* Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **396**, 580-584 (1998).
8. Small, S., Keerthivasan, G., Huang, Z., Gurbuxani, S. & Crispino, J.D. Overexpression of survivin initiates hematologic malignancies in vivo. *Leukemia*.
9. Wobser, M.*, et al.* Complete remission of liver metastasis of pancreatic cancer under vaccination with a HLA-A2 restricted peptide derived from the universal tumor antigen survivin. *Cancer Immunol Immunother* **55**, 1294-1298 (2006).
10. Ryan, B.M., O'Donovan, N. & Duffy, M.J. Survivin: a new target for anti-cancer therapy. *Cancer Treat Rev* **35**, 553-562 (2009).
11. Tsuruma, T.*, et al.* Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. *J Transl Med* **2**, 19 (2004).
12. Ahn, B.C.*, et al.* Potent, tumor-specific gene expression in an orthotopic hepatoma rat model using a Survivin-targeted, amplifiable adenoviral vector. *Gene Ther*.
13. Blanc-Brude, O.P.*, et al.* IAP survivin regulates atherosclerotic macrophage survival. *Arterioscler Thromb Vasc Biol* **27**, 901-907 (2007).
14. Shaposhnik, Z., Wang, X. & Lusis, A.J. Arterial colony stimulating factor-1 influences atherosclerotic lesions by regulating monocyte migration and apoptosis. *J Lipid Res* **51**, 1962-1970.
15. Tedgui, A. & Mallat, Z. Apoptosis as a determinant of atherothrombosis. *Thromb Haemost* **86**, 420-426 (2001).
16. Zeis, M.*, et al.* Generation of cytotoxic responses in mice and human individuals against hematological malignancies using survivin-RNA-transfected dendritic cells. *J Immunol* **170**, 5391-5397 (2003).
17. Siegel, S., Wagner, A., Schmitz, N. & Zeis, M. Induction of antitumour immunity using survivin peptide-pulsed dendritic cells in a murine lymphoma model. *Br J Haematol* **122**, 911-914 (2003).
18. Adida, C.*, et al.* Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am J Pathol* **152**, 43-49 (1998).
19. Blanc-Brude, O.P.*, et al.* Inhibitor of apoptosis protein survivin regulates vascular injury. *Nat Med* **8**, 987-994 (2002).
20. Groeneweg, M.*, et al.* Modification of LDL with oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) results in a novel form of minimally modified LDL

that modulates gene expression in macrophages. *Biochim Biophys Acta* **1781**, 336-343 (2008).
21. Breckpot, K.*, et al.* Lentivirally transduced dendritic cells as a tool for cancer immunotherapy. *J Gene Med* **5**, 654-667 (2003).
22. Goossens, P.*, et al.* Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. *Cell Metab* **12**, 142-153.
23. Esslinger, C.*, et al.* In vivo administration of a lentiviral vaccine targets DCs and induces efficient CD8(+) T cell responses. *J Clin Invest* **111**, 1673-1681 (2003).
24. Dullaers, M.*, et al.* Induction of effective therapeutic antitumor immunity by direct in vivo administration of lentiviral vectors. *Gene Ther* **13**, 630-640 (2006).
25. Hansson, G.K. & Nilsson, J. Vaccination against atherosclerosis? Induction of atheroprotective immunity. *Semin Immunopathol* **31**, 95-101 (2009).
26. Riley, E., Dasari, V., Frishman, W.H. & Sperber, K. Vaccines in development to prevent and treat atherosclerotic disease. *Cardiol Rev* **16**, 288-300 (2008).
27. van Puijvelde, G.H.*, et al.* Induction of oral tolerance to oxidized low-density lipoprotein ameliorates atherosclerosis. *Circulation* **114**, 1968-1976 (2006).
28. Taleb, S., Tedgui, A. & Mallat, Z. Adaptive T cell immune responses and atherogenesis. *Curr Opin Pharmacol* **10**, 197-202.
29. van Wanrooij, E.J.*, et al.* Vaccination against CD99 inhibits atherogenesis in low-density lipoprotein receptor-deficient mice. *Cardiovasc Res* **78**, 590-596 (2008).
30. Hauer, A.D.*, et al.* Vaccination against VEGFR2 attenuates initiation and progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* **27**, 2050-2057 (2007).

Chapter 5

Macrophage heterogeneity: relevance and functional implications in atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease involving many cell types with a well-accepted key role for macrophages. A wide array of different properties and functional characteristics are attributed to macrophages present in the atherosclerotic plaque. As an increasing body of evidence strengthens the consensus that macrophages comprise a heterogeneous population, several co-existing subtypes with diverse, even opposing specialties have already been described in fields like parasitology, tumour biology and metabolic disorders. However, macrophage heterogeneity within atherosclerotic lesions has not been studied in detail yet. In this review we will introduce the characteristics of macrophage subsets in other pathologies and address the presence and possible roles of distinct macrophage subtypes in the rapidly evolving field of atherosclerosis. Finally, we make an effort to relate these subtypes to disease progression and explore a number of opportunities for novel diagnostic and therapeutic approaches.

Introduction

Atherosclerosis is a multifactorial disease that afflicts the medium and large sized arteries of the body. Formerly perceived as a mere lipid storage disease, the treatment of atherosclerosis in patients has focused mainly on the lowering of plasma cholesterol. Although effective lipid lowering can be achieved pharmaceutically, cardiovascular disease (e.g. myocardial infarction, stroke) still constitutes the main cause of mortality in modern societies. Since these acute clinical events are largely attributable to complications of atherosclerotic lesions, novel strategies for prevention and management of atherosclerosis are in high demand. From this perspective, the fundamental role of inflammation as a mediator of all stages of atherogenesis is being appreciated to an ever increasing extent. Not only does the chronic inflammatory response associated with atherosclerosis act alongside well-known environmental and genetic risk factors to induce lesion initiation, it also promotes progression and ultimately leads to plaque rupture by reducing lesion stability[1,2]. Consequently, extensive scientific efforts have been made over the past decade to illuminate the inflammatory processes that underlie atherosclerosis development at the molecular and cellular level.

Atherosclerosis, as an inflammatory condition, involves many cell types and mediators of the immune system. In this regard, monocyte-derived macrophages are imperative as these immune cells partake in innate and adaptive (Th1 and Th2-mediated) immune responses, which are both intricately engaged in atherogenesis[2]. Macrophages do not only form one of the main cellular constituents of the lesions (e.g. as foam cells), they also serve as a major source of inflammatory mediators. Thereby they determine the inflammatory equilibrium (i.e. the balance between pro- and anti-inflammatory factors) during atherogenesis, greatly impact the activation, migration and survival of other cells in the plaque and ultimately affect plaque stability. As such, macrophages are generally accepted as key players in the pathophysiology of atherosclerosis that profoundly shape atherosclerotic plaque development. The morphology of macrophages in atherosclerotic lesions can vary dramatically, from a large quiescent lipid-laden foam cell to a small active inflammatory cell. Recent work in the field of innate immunity has led to the identification of macrophage subsets based on their immune polarization. These subsets portray divergent characteristics and identification of specific markers and their functional role *in vivo* has recently gained strong interest in different fields of inflammatory disease. Knowledge on the in vivo contribution of different macrophage populations to the development of atherosclerosis however is scarce. Nevertheless, based on their distinct physiological roles, macrophage subsets are expected to highly determine plaque composition, stability and consequently clinical outcome. Taking approaches with experimental animal models as lead, in this review we will highlight the typical features of macrophage subsets and discuss their relevance and

contribution in a number of pathologies. Moreover, we speculate on the functional characteristics of macrophage subsets in atherosclerosis and address opportunities that may arise in using macrophage subsets for therapeutic targeting and the diagnosis of atherosclerosis.

Macrophage heterogeneity

Cells of the mononuclear phagocyte lineage (i.e. monocytes, macrophages) are characterized by a substantial degree of heterogeneity[3,4]. Especially macrophages are known to express functionally different phenotypes in response to tissue-derived signals and the immunological micro-environment, thus skewing them towards their many tasks in homeostasis, host defence and pathology[4-9]. Furthermore, macrophages display notable plasticity, as they are capable of rapidly switching between activation states in response to a specific incentive[9-11]. Accordingly, under inflammatory conditions, macrophage populations may initially partake in promoting inflammation and later contribute to its resolution[12-14]. Over the last decade, a conceptual framework has been devised and gradually expanded to account for the polarized functional properties of different macrophage populations. In reflection of the Th1 and Th2 nomenclature in lymphocytes, polarized macrophage subsets are mostly referred to as M1 and M2 macrophages[5,6,15].

Classically activated or M1 macrophages are elicited through stimulation with the Th1-cytokine interferon-γ (IFN-γ) alone, through a concomitant stimulus of IFN-γ with microbial products, such as lipopolysaccharide (LPS) or by activation with the Th1 cytokine tumour necrosis factor (TNF). Although these cells show extensive morphological diversity based on their tissue localization, M1 cells share the ability to secrete large quantities of pro-inflammatory cytokines, such as TNF, interleukin (IL)-1β, IL-6, IL-18 and IL-12[5,13]. Additionally, they produce high levels of anti-microbial effector molecules (reactive oxygen and nitrogen species; ROS, RNS). These qualities allow M1 macrophages to act as potent inducers and effectors of polarized type I immune responses, consequently associating them with resistance to pathogens and tumours as well as tissue destruction[16-19]. Recently, Martinez et al. have suggested a further subdivision of the M1 subset in M1a and M1b macrophages to distinguish between classical and innate activation types [13]. Whereas the latter phenotype is said to be induced through ligation of for instance Toll-like receptors (TLR) by so-called Pathogen-Associated Molecular Patterns (e.g. LPS) and somewhat resembles the classical activation profile, the different phagocytic properties and inability to produce functional amounts of IL-12, sets it apart from classically activated M1a macrophages[20,21]. However, in our view, macrophages derived through these activation pathways still need to be characterized more thoroughly.

M1	M2a	M2b	M2c
IFN-γ + LPS or TNF-α	IL-4, IL-13	IC + IL-1β or TLR-ligands	IL-10 (TGF-β, glucocorticoids)
IL-10^{low}	IL-23^{low}	IL-23^{low}	IL-23^{low}
TNF	TGF-β	TNF	TGF-β
IL-1β		IL-1	IL-1Ra
IL-6		IL-6	
IL-18			
NO, ROS (iNOS)	polyamines (Arg-1)		
CCL2, CCL3, CCL4, CCL5	CCL17, CCL18, CCL22, CCL24	CCL1	CCL18
MHC-II	MR	CD86	MR
CD86	decoy IL1-RII		CD163
CD80	SR		SR
IL-1RI			
TLR2/ TLR4			

Table 5.1: Simplified overview of the phenotypical characteristics of M1 and M2 macrophages. *Abbreviations: IFN-γ, interferon-γ; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-α; IL, interleukin; IC, immune complexes; TGF-β, transforming growth factor-β; GC, glucocorticoids; NO, nitric oxide; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; Arg-1, arginase-1; IL-1Ra, IL-1 receptor antagonist; CCL, chemokine C-C motif ligand; MHC-II, major histocompatibility complex class II; CD, cluster of differentiation; IL-1RI, IL-1 receptor I; MR, mannose receptor; SR, scavenger receptor.*

In recent years, the term M2 has served as a generic name indicating the various forms of alternatively activated or anti-inflammatory macrophages[4,22]. As opposed to classical activation by IFN-γ, alternative activation of macrophages is induced by several different stimuli and a subdivision of M2 cells has been made accordingly. M2a cells are generated by stimulation with the Th2-cytokines IL-4 and IL-13[23]; M2b cells correspond to concomitant stimulation with immune complexes and either LPS or IL-1β (initially termed type II activation)[24,25], whereas M2c macrophages are triggered by IL-10 (and to some extent by transforming growth factor-β (TGF-β) or glucocorticoids). Functionally, M2 macrophages are integrated in type II immune responses, accounting for the attenuation of excessive Th1-mediated inflammation, tissue remodeling, allergy, tumour progression and host defence against extracellular parasites[5,7,15,22]. Although the diversity between M2 macrophages is apparent[14,26], usage of this term is justified by the fact that various forms of M2 macrophages do share a number of phenotypical similarities[4]. Generally, M2 cells produce low amounts of pro-inflammatory factors (e.g. IL-12), in contrast to an increased production of anti-inflammatory mediators such as IL-10 and the pro-fibrotic factor TGF-β. However, in this regard the M2b subset stands out by the fact that it is characterized by a higher inflammatory cytokine production (e.g. TNF, IL-1 and IL-6) and does not show induction of arginase-1 (Arg-1) or TGF-β. On the other hand, these cells still clearly portray a high IL-10 and low IL-12 profile, which warrants their M2 classification[19]. M2 macrophages additionally display high levels of scavenger receptor activity and express a chemokine repertoire that is dissimilar from

that of M1 macrophages[4,27]. Finally, in M2a macrophages as opposed to M1 cells, the arginine metabolism is generally shifted to the production of proline and polyamines through activity of the profibrotic enzyme Arg-1. This shift inversely correlates with inducible nitric oxide synthase (iNOS)-mediated nitric oxide (NO) production[28]. A simplified summary of the characteristics of each respective phenotype is presented in Table 5.1.

Macrophage heterogeneity in health and disease

Although the contribution of different macrophage subsets to atherosclerosis is very scarcely studied[29,30], the concept of macrophage heterogeneity has already been well explored in other pathologies with macrophage-related origins (i.e. infectious disease, cancer and metabolic disorders). Logically, knowledge from lessons learnt in other fields might be useful when applied to atherosclerosis research, provided that the underlying mechanism by which macrophage dysfunction causes morbidity is taken into account[31]. Often, when conditions manifest with a disturbed inflammatory balance, immune cells (e.g. macrophages) are either unable to react to environmental changes or by contrast exaggerate their response to such events. In these situations, one macrophage subset may be favoured over another. It is very likely that such an imbalance also plays a role in the pathogenesis of atherosclerosis, making it very relevant to study the dynamics in subset distribution throughout the course of this disease. Thereby, we might better appreciate how each subset contributes to the many specialized macrophage functions in the atherosclerotic plaque.

Macrophage heterogeneity in infectious disease

Regulation of the delicate balance between M1 and M2 type polarization has been shown to be critical in host defence against many types of pathogens. Based on the analysis of host transcriptomes in several studies, Jenner et al. identified a set of genes that together comprise the 'common host response' of innate immune cells in reaction to a range of bacteria, viruses and fungi[32]. Especially monocyte-derived macrophages seem to develop a common pattern of gene expression when challenged by bacteria[33]. Interestingly, Benoit et al. reported more recently that this shared response to bacterial infection is mainly associated with increased expression of genes that are involved in polarization towards a functional M1 program. More specifically, these include the genes that encode for TNF, IL-6, IL-12, iNOS and monocyte chemotactic protein-1 (MCP-1), also known as chemokine ligand 2 (CCL2)[34]. Up-regulation of these genes partially accounts for the enhanced microbial killing activity that is regarded as a typical characteristic of M1 macrophages. Thus, by augmenting the immediate resistance to bacteria, M1 polarization promotes immunity and serves a protective role in host defence during acute infectious disease. However, an extended period of M1 skewing

may lead to a deleterious outcome for the host if not kept in check. This is illustrated by the correlation between the amount of circulating M1-type cytokines and increased sepsis severity in a human population[35]. In their review, Benoit et al. further provide a detailed account of the approaches that several bacterial pathogens have developed to hamper the M1 polarization of macrophages in an attempt to endure in their surroundings. Different strategies include inhibition of the oxidative microbicidal capacity of macrophages (NO release), inhibition of the expression and secretion of M1-type cytokines (primarily IL-12 and TNF), the release of virulence factors that interfere with M1-associated cellular signaling pathways and finally, direct promotion of M2 polarization[34]. Skewing of macrophages towards an M2 phenotype is associated with the continual presence of bacteria in tissues and the conversion of some infectious diseases to a chronic state. For instance, replication of *Coxiella burnetii,* the causative agent of Q fever, was shown to be related to macrophages expressing a M2 signature that featured up-regulation of Arg-1, YM-1 and the mannose receptor (MR) and down-regulation of iNOS and M1-type cytokines. Activation by IFN-γ however, inhibits replication of this pathogen and reprograms macrophages towards M1[36-38]. Additionally, Whipple's disease, which is caused by *Trophyrema whipplei*, has also been linked to M2 polarization. Whereas this pathogen is eradicated in an M1-driven immunological micro-environment, it actively replicates in a M2 setting[39]. Notably, these are only a few examples of the growing list of pathogens that take advantage of changes in macrophage activation profiles.

Over the last few years, studies on macrophage function in parasite infections have also yielded great knowledge on the existence and function of different macrophage subsets in disease states[40]. In general, infection of a host organism with both intracellular and extracellular parasites triggers a specific inflammatory response. As a first line of defence preceding adaptive immunity, macrophages mediate both anti-parasitic immunity and parasite clearance by phagocytosis and immunosuppression. A pro-inflammatory response by M1 macrophages, targeted at parasite elimination, is crucial for limiting parasite growth during the acute phase of the infection. After this initial immune reaction, a type II inflammatory response ensues aiming to resolve the inflammatory process. Any disturbance of the equilibrium between these two phases will consequently worsen the outcome of the infection. Whereas lack of the initial M1 response would result in uncontrolled parasite colonisation, an insufficient M2 response would lead to more efficient parasite killing that is however accompanied by excessive systemic inflammation. The subsequent anaemia and liver damage by ROS might eventually result in host death. The infection with *Trypanosoma congolense*, the African parasite responsible for sleeping sickness, exemplifies the influence this fragile balance of macrophage polarization has on parasite infection and even how this parasite can exploit these changes in the inflammatory response. Upon infection, this extracellular parasite will primarily settle in the liver, spleen and brain. It is covered by variant-specific surface glycoproteins (VSG) that are very immunogenic and cause a T-

cell dependent B-cell response. However, by frequently transforming their molecular structure, these VSGs avoid targeting by the adaptive immune response and make vaccination against this parasite impossible[41]. Trypanosomes can be phagocytosed upon binding of antibodies to VSG, but this binding simultaneously releases these molecules to the circulation as soluble VSG (sVSG). Initially, sVSG modulate macrophage function towards systemic macrophage activation with M1 characteristics, which is sustained by T-cell-derived IFN-γ and characterized by production of the trypanotoxic agents NO and TNF. Following this early response, a transition towards a type II response takes place that mainly involves M2a macrophages and is characterized by high plasma levels of IL-10, IL-4 and IL-13. The end result is tolerance towards the parasite with long host survival and limited tissue damage[42-44]. However, C57BL/6 mice lacking either TNF[45] or IL-10[46] signaling are both susceptible to trypanosomes and die upon infection, by excessive parasite growth or by an IFN-γ mediated shock syndrome respectively[47]. Concluding, fighting bacterial and parasitic infections necessitates an adequate regulation of the balance between M1 and M2 macrophages to ensure proper microbial killing. Increasing our knowledge on this part could eventually lead to more efficient ways to treat infections. Although selectively promoting or inhibiting M1 or M2 phenotypes at the right moment is likely to enhance the effectiveness of the inflammatory response, one should be aware of the risks involved with intervening in this delicate balance. Overstimulation of the M1 phenotype for a prolonged period will result in excessive tissue damage. Preventing an appropriate M2 macrophage response from counteracting M1-mediated inflammation might pose a similar hazard. Promotion of the M2 phenotype on the other hand will probably facilitate the survival and growth of pathogens. Additionally, the way in which the M1/M2 balance shifts during the course of different infections may prove another pitfall. Temporal variability in the macrophage response to distinct micro-organisms would hamper large-scale application of macrophage-based approaches. Furthermore, this would necessitate detailed knowledge of the macrophage kinetics in certain types of infection if successful timing of such a therapeutic approach were to be achieved.

Tumour associated macrophages

Another model illustrating the relevance of the macrophage M1/M2 paradigm in pathologic situations concerns the macrophages that are found within tumours[48]. A tumour micro-environment not only exists of malignant cells but also includes stroma and cells belonging to the immune system. The latter are part of a system of immune surveillance that aims to eliminate tumour cells through innate and adaptive responses[49]. Tumour cells evade this targeting through their genetic variability, active immune suppression and secretion of tolerogenic effectors. Because of their contribution to both the targeting and the protection of the tumour cells, tumour associated macrophages (TAMs) are a great example of the possible implications of macrophage heterogeneity in vivo[8,50,51]. Moreover, their ambivalent functional

characteristics clearly show the caveats to be dealt with when contemplating macrophage directed therapies[52].

Macrophages that are attracted to tumour sites by factors like M-CSF and CCL2[53,54], are initially cytotoxic through secretion of ROS and slow down tumour cell proliferation. This M1 phenotype induces an inflammatory environment that attracts and activates cells of the adaptive immune system[55,56]. However, with sustained activation, these cells increase the risk of DNA damage and tissue structure abnormalities, thus promoting carcinogenesis[57]. However, tumour cells that are lysed by these M1 macrophages release factors including TGF-β and sphingosine-1-phosphate, which reprogram the macrophages towards an alternatively activated phenotype[58]. Subsequently, these M2 macrophages will temper the inflammatory response and create a more tolerogenic environment by secreting IL-10 and TGF-β[58]. Moreover, these cells mediate cell proliferation and tissue remodelling through arginase induction and tumour invasiveness through secretion of chitinase-like proteins, further contributing to tumour development. Another important feature of the alternatively activated TAMs is their role in the formation and remodeling of new blood vessels in the tumour area by releasing both pro-angiogenic factors such as vascular endothelial growth factor (VEGF**)**, TNF, IL-8 and basic fibroblast growth factor (bFGF) as well as angiogenesis modulating enzymes such as matrixmetalloproteinase (MMP)-2, MMP-7, MMP-9, MMP-12 and cyclooxygenase (COX)-2[59]. As the rapid tumour growth demands increasing perfusion to prevent hypoxia and subsequent necrosis, angiogenesis is essential for disease progression[60].

The formation of tumour promoting TAMs has been linked directly to the nuclear factor-κB (NF-κB) signal transduction pathway, which activates transcription factors that play an essential role in regulating inflammation and immunity[61]. Especially the NF-κB subunit p50 has been identified as an important regulator of macrophage polarization in tumours. The observation that TAMs are defective in their production of the NF-κB target gene IL-12 but rather show higher levels of IL-10 indicated that a regulation at the level of NF-κB could be expected[62]. It was shown that p50 can form inhibitory homodimers that are able to bind the NF-κB consensus binding sites, thus repressing transcription of a subset of NF-κB-dependent inflammatory genes[63]. Indeed, TAMs show tolerance toward both LPS and other pro-inflammatory signals as indicated by a defective IL-12, IL-6 and TNF expression and relatively enhanced IL-10 expression[64]. Moreover, TAMs were characterized by massive nuclear overexpression of p50. Interestingly, the anti-inflammatory phenotype of TAMs was reversed in macrophages lacking p50 and both p50-deficient mice and wildtype mice that received p50-deficient bone marrow displayed a delayed tumour progression with increased survival[64]. Hence, activation of the NF-κB subunit p50 appears to induce an M2 phenotype in TAMs. Continuing on the role of NF-κB signaling in regulating TAM function, it was more recently shown that the main kinase mediating NF-κB activation, inhibitor of NF-κB kinase 2 (IKK2 or IKKβ), inhibits M1 differentiation of macrophages by affecting signal

transducer and activator of transcription-1 (STAT1) signaling. Consequently, macrophage-specific deletion of IKK2 led to a more pronounced M1 phenotype, characterized by high IL-12, iNOS and MHC-II expression and low levels of IL-10[65,66]. This phenotypic shift resulted in macrophages that were better capable of actively eliminating tumour cells, both directly by enhanced production of oxygen radicals and indirectly through IL-12-dependent natural killer (NK)-cell anti-tumour activity[66]. Thus, NF-κB activation is intricately involved in the induction of anti-inflammatory tumour repressive TAMs at different levels.

Since high TAM density in tumours is correlated with a poor prognosis[59,67], blocking the attraction of macrophages to the tumour might seem a promising therapeutic target. Indeed, in experimental models this approach showed to inhibit further tumour growth[68-71]. However, such a strategy would also rule out the potential of attracting more M1 polarized macrophages to engage in tumour cell lysis and promote an anti-tumour immune reaction. Therefore, induction of macrophage polarization towards an M1 phenotype would be a more desirable treatment option than merely interfering with macrophage attraction to tumours. For now, the major challenge will be to find techniques that locally and specifically skew macrophages to the desired phenotype. Until such methods are developed, it remains unclear if any phenotype promoting or targeting treatment will suffice as a stand-alone therapy or will merely serve as an adjuvant therapeutic option.

Macrophage heterogeneity in obesity and insulin resistance

In recent years, mounting evidence suggests that low-grade inflammation contributes to the development of obesity and progression to insulin resistance[72]. More specifically, recent work has recognized adipose tissue macrophages (ATM) as the main source of pro-inflammatory mediators in adipose tissue and has correlated their function with the degree of insulin resistance[73]. Interestingly, in lean mice, resident ATMs exhibit characteristics of M2a macrophages with expression of Arg-1 and IL-10[74]. From a functional perspective, this IL-10 production was shown to protect adipocytes from TNF-induced insulin resistance[74], advocating these M2 macrophages as being protective in obesity. Rather than altering macrophage activation state, the induction of obesity through a high fat diet superimposes a new population of macrophages to adipose tissue that possess an M1-polarized phenotype with expression of pro-inflammatory markers such as iNOS and TNF[75]. With progressive obesity, these M1 macrophages accumulate in adipose tissue through recruitment by MCP-1 dependent mechanisms. Absence of MCP-1 or its receptor CCR2 abolishes migration of macrophages into adipose tissue, thereby preventing adipose tissue inflammation and improving insulin resistance[76,77].

The M2 phenotype of ATMs in lean mice was subsequently shown to be maintained by the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). Induction of

alternatively activated macrophages by IL-4 was reduced in the absence of PPARγ and was accompanied by reduced Arg-1 activity, decreased suppression of IL-6 and inhibition of fatty acid oxidation in macrophages. Furthermore, macrophage-specific deletion of PPARγ in vivo reduced the M2 phenotype of ATMs, impaired adipocyte function by reducing mitochondrial oxidation and ultimately resulted in enhanced obesity and insulin resistance[78]. In line with these findings, Hevener et al. showed that macrophage-specific PPARγ deficiency propagates impaired insulin sensitivity in muscle, liver and adipose tissue and worsens insulin resistance upon high fat feeding of mice[79]. Thus, PPARγ is necessary for sustaining the M2 phenotype in adipose tissue of lean individuals and thereby protects against obesity and insulin resistance.

Remarkably, contrary to ATMs, disruption of PPARγ does not affect Kupffer cells, the liver's resident macrophages. In these cells, it is the transcriptional activation of another nuclear receptor known as PPARδ by mono-unsaturated fatty acids that induces an alternative activation state. Analogous to PPARγ deficiency, abrogation of PPARδ impairs the alternative activation of Kupffer cells, thereby causing hepatic dysfunction, diet-induced insulin resistance and obesity[80]. Similar observations were described by Kang et al.[81], demonstrating that myeloid-specific PPARδ deficiency leads to insulin resistance and severe steatohepatitis. Hence, macrophage polarization in adipose tissue and liver is chiefly regulated by PPARγ and PPARδ respectively and provides an important incentive in the pathogenesis of metabolic diseases. Whereas the anti-inflammatory phenotype displayed by resident ATMs in healthy conditions appears to be protective, the obesity-induced recruitment of pro-inflammatory M1 macrophages alters the macrophage polarization balance in adipose tissue and aggravates insulin resistance.

The macrophage as central player in atherosclerosis initiation and progression

From the above it is evident that macrophage polarization is a main determinant in the aetiology of many different diseases. In terms of atherosclerosis, this is no different. The major initiating events in atherosclerosis development encompass endothelial dysfunction and modification of low-density lipoprotein (LDL)[82]. Aberrant blood flow renders specific areas of the vessel wall more susceptible to atherogenesis, since the altered haemodynamics increase permeability of the endothelial barrier to macromolecules such as LDL[1]. These molecules subsequently accumulate in the subendothelial matrix, where they undergo modification through oxidative or enzymatic processes. Retention of these modified lipoproteins exerts a pro-inflammatory stimulus on the micro-environment, thereby promoting expression of a range of adhesion molecules (e.g. vascular cell adhesion molecule 1; VCAM-1), chemotactic proteins (e.g. MCP-1) and growth factors (e.g. macrophage-colony stimulating factor; M-CSF) by the overlying endothelium[83]. This in turn mediates

leukocyte recruitment and monocyte migration into the intima at lesion prone sites [1]. Upon M-CSF-induced differentiation, monocyte-derived macrophages will start to internalize substantial amounts of modified LDL (mLDL) via scavenger receptors (SR)[1,83,84]. Although this process of foam cell formation may at first serve a protective role in removing pro-inflammatory modified lipids and apoptotic cell debris from the vessel wall, the increasing build-up of these cells ultimately leads to the development of fatty streaks. The progression of a clinically insignificant fatty streak to a more complex lesion is hallmarked by the migration of smooth muscle cells (SMC) from the media to the subendothelial space of the vessel wall[1,83]. In conjunction with a continuous influx of immune cells, these SMCs can proliferate within the lesion, contribute to foam cell formation but primarily secrete extracellular matrix components. Concurrently, apoptotic foam cells that are improperly scavenged will contribute their lipid-filled contents to the growing necrotic core of the plaque[82]. Ultimately, an advanced lesion is formed, in which the lipid-rich necrotic core is shielded from the lumen by a fibrous cap of SMCs and extracellular matrix. In disease progression to clinical manifestation, rupture of this fibrous cap is generally thought to elicit thrombosis, as exposure of plaque lipids and tissue factor to blood components will consequently initiate the coagulation cascade[1,85]. The physical integrity of the fibrous cap therefore is vital for plaque stability. Interestingly, the balance between trophic and degenerative factors that determine fibrous cap thickness can be profoundly affected by both cellular and humoral factors of immunity. While IFN-γ, for instance, inhibits extracellular matrix production by SMCs, plaque macrophages enable the degradation of extracellular matrix proteins by the production of MMPs[82,86]. As such, the macrophage is acknowledged as a key player in atherosclerotic in the regulation of plaque development and stability (see Figure 5.1).

Macrophage heterogeneity in atherosclerosis

As detailed above, the functional characterization of macrophage heterogeneity in numerous different macrophage-related pathologies has been ongoing for several years now. Based on their inflammatory characteristics, M1 macrophages can be expected to promote atherosclerosis development, while M2 macrophages in general may be considered to be protective in this regard. However, to this point, the functional contribution of individual macrophage subsets to atherosclerosis has barely been studied, despite the fact that macrophage heterogeneity in human atherosclerotic lesions was already demonstrated early on. Using differentiation markers and stainings for lipids and lysosomal phosphatase, van der Wal and co-workers reported that more centrally localized macrophages in lesions have more differentiated and matured characteristics, than their counterparts in the superficial layers of these plaques[87]. This

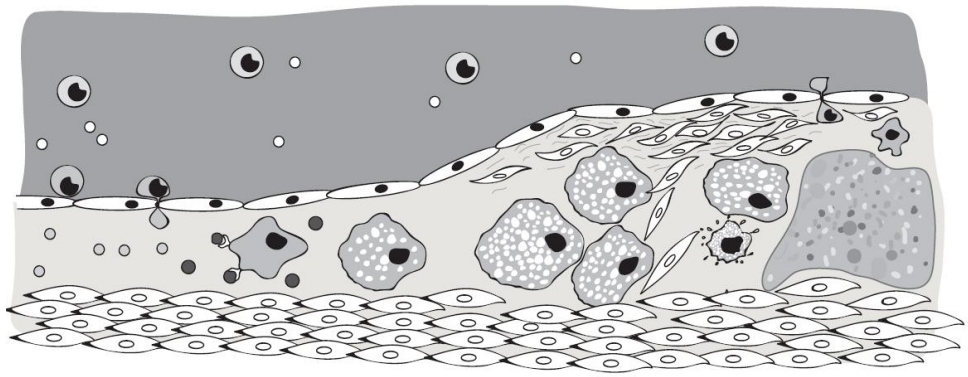

Figure 5.1: Macrophages are paramount to plaque development:

A schematic outline of the lumen and vessel wall is shown in which, from left to right, the development of an atherosclerotic plaque is depicted. Early phases of the disease involve the subendothelial retention and accumulation of LDL. These particles are subsequently modified and induce endothelial activation and chemokine secretion. In response, attracted monocytes migrate into the vessel wall where they differentiate into macrophages. These macrophages commence continual scavenging of these modified lipoproteins, which transforms them into foam cells that characteristically contain intracellular lipid droplets. Persisting foam cell accumulation eventually results in the formation of a fatty streak. Macrophages in atherosclerotic lesions also secrete lipid modifying enzymes, which promote further LDL oxidation, and chemokines and cytokines that control the inflammatory balance and regulate recruitment, activation and proliferation of cells. Upon further progression of atherogenesis, T-cells are attracted and activated which propagate the chronic inflammatory state of the lesion. When foam cells die through apoptosis, improper scavenging of these cells leads to secondary necrosis and the release of cellular debris (i.e. scavenged lipids) to the growing lipid-rich necrotic core of the lesion. As smooth muscle cells proliferate and migrate, they form a fibrotic cap that encloses the lesion and its necrotic core. In time, inflammatory factors compromise the integrity of this fibrous cap, resulting in thrombus formation when rupture of the fibrous cap exposes thrombogenic plaque contents to the blood. This process of rupture is promoted by the local recruitment of macrophages, producing matrix-degrading enzymes. Abbreviations: LDL, low density lipoproteins.

implied functional differences between macrophage populations within the same lesions. Similarly, using a set of macrophage specific monoclonal antibodies, it was shown that macrophages in murine atherosclerotic lesions show a large degree of heterogeneity as well[88]. More recently, Waldo et al. confirmed the heterogeneity of human macrophages in atherosclerotic lesions yet again by using CD14 positivity to discriminate between subsets[29]. Thus, heterogeneity of macrophages has been well established in human and mouse atherosclerotic lesions. However, the amount of data that provide a direct functional link between the M1 and M2 subsets and atherosclerotic lesions is limited.

Recently, Bouhlel et al.[30] were the first to describe the expression of M1 and M2 markers in atherosclerotic lesions. Concretely, they demonstrated M1-associated marker genes such as MCP-1, IL-6 and TNF and MR, CD163, IL-10 and CCL18, markers of M2 macrophages, to be expressed in human atherosclerotic lesions. Moreover, they

established a direct correlation between the expression of M2 markers and that of PPARγ, indicating a role for PPARγ in regulating M2 differentiation in atherosclerosis, similar to what was reported previously in murine diet-induced obesity (see above)[78]. Notably, through cell cultures they were able to confirm that PPARγ activation polarizes human macrophages towards an M2 phenotype. Interestingly, patients treated with PPARγ agonists showed a more pronounced M2 phenotype in their circulating mononuclear cells, whereas M2 marker expression in atherosclerotic lesions did not change. Although certain questions regarding the therapeutic benefit of this approach remain unanswered, this paper offers an interesting functional perspective by identifying the expression and localization of certain subset markers in atherosclerosis.

More recently, Boyle et al. reported intraplaque haemorrhage, a main contributor to lesional development and instability, to induce a novel protective macrophage phenotype in atherosclerotic plaques of human coronary arteries[89]. Primarily defined by high levels of the scavenger receptor CD163, these macrophages use this surface molecule to bind and clear haemoglobin-haptoglobin complexes from the vessel wall. As a result, $CD163^{high}$ macrophages secrete a number of anti-oxidant and anti-inflammatory mediators (e.g. IL-10 and haeme oxygenase-1) in an attempt to counter the atherogenic effects of plaque haemorrhage[89]. However, in spite of its beneficial qualities, this phenotype is by no means fully atheroprotective as is illustrated by the use of post-mortem specimens of culprit lesions in this study. This can be partly explained by the fact that even lesions with substantial haemorrhage predominantly featured pro-inflammatory macrophages, whereas plaques devoid of haemorrhage lacked $CD163^{high}$ macrophages altogether. As such, the relatively small size of the $CD163^{high}$ subpopulation seems to limit its impact on the surrounding tissue. Taken together, these data imply that CD163 might in fact be a suitable marker for high-risk atherosclerotic plaques. This notion is confirmed by Papaspyridonos et al.[90], who found the expression of CD163 to be upregulated almost sevenfold in unstable plaque regions as compared to stable areas. Interestingly, the presence of neutralizing IL-10 antibodies prevented the development of $CD163^{high}$ macrophages in vitro, hereby showing a role for IL-10 in inducing this phenotype. Moreover, glucocorticosteroids have also been found to elicit CD163, be it to a lesser extent than haemoglobin-haptoglobin complexes or IL-10, which further enhances the analogy to the M2c subtype[91]. Unfortunately, to our knowledge, this is the only report to date that directly associates an M1/M2 subset marker with the functional phenotype (i.e. stability) of an atherosclerotic lesion.

Macrophage subset effector function and atherosclerosis

From a theoretical perspective and knowing the many specialized functions macrophages exert in all stages of atherogenesis, it can be expected that specific features of the M1 and M2 polarized macrophage subsets described before in this review contribute differently to plaque development. First of all, a range of cytokines

that has been associated to macrophage polarization (see table I) has been the subject of extensive atherosclerosis research (as reviewed by Tedgui and Mallat, Kleemann et al.)[92,93]. In general, cytokines that are specifically produced by M1 macrophages have a proatherogenic effect. Using knockout, transgenic, treatment or inhibitory approaches, it is firmly established that cytokines such as TNF, IL-1 and IL-12 promote disease progression in different animal models of atherosclerosis. In contrast, M2 associated cytokines are either anti-atherogenic (e.g. IL-10), or combine anti-atherogenic characteristics with profibrotic properties (e.g. TGF-β). These cytokine data are in line with the concept that M1 macrophages promote atherosclerosis, whilst M2 macrophages inhibit plaque growth and mediate plaque stability.

For chemokine patterns associated with macrophage polarization [4], the story is more complex. M1 inducers (e.g. IFN-γ and TLR-ligands) usually act through signal transduction pathways such as NF-κB and STAT-1 to evoke transcription of inflammatory chemokines (CCL2, 3, 4 and 5 amongst others)[94,95]. Moreover, both CCL5 (RANTES) and CCL2 have been studied to some extent in atherosclerosis[96]. While CCL5 blockade reduces atherosclerosis[97], inhibition of its two receptors (i.e. CCR1 and CCR5) has opposing effects. CCR5 acts pro-atherogenic as genetic deletion of this receptor reduces atherogenesis and inhibits Th1 responses[98-100], while in contrast CCR1-deficient mice show accelerated atherosclerosis, with IFN-γ being an important mediator [101,102]. CCL2 and its receptor CCR2 seem to have a more clear-cut role in promoting atherosclerosis[103-105] by regulating the specific recruitment of inflammatory monocytes to atherosclerotic lesions[106]. Interestingly, M2 stimuli (e.g. IL-4, IL-13 and IL-10) interfere with the general expression of M1-associated chemokines through inhibition of the same pathways that mediate M1 responses. Moreover, these signals elicit expression of a set of chemokines that is linked to type II immune responses[4]. As such, M2a macrophages display expression of CCL17, 18, 22 and 24 for instance[107,108], whereas both the M2a and M2c subsets express CCL18[109]. Many other chemokines are currently still in need of better association with particular macrophage subsets or have yet to be investigated for their role in atherosclerosis development in general.

In accordance with the atherogenic function of M1 macrophages and the putative atheroprotective effect of M2 macrophages, the ways in which these subtypes utilize the common substrate arginine also influence lesion fate. As mentioned before, M2 skewing induces Arg-1 expression that enables the conversion of arginine to collagen precursors such as ornithine and subsequently proline and polyamines. In macrophages, this diverts arginine metabolism away from M1-associated iNOS-derived NO synthesis Hereby, M2 macrophages may contribute to enhancing the physical integrity of the fibrous cap, thus promoting plaque stability, and reduce atherogenesis. This was recently substantiated by genetic analysis of two rabbit strains with high and low susceptibility for atherosclerosis, showing that the rabbits which had reduced atherosclerosis development were characterized by elevated levels of Arg-1 activity, regulated through a polymorphism in the 3'-untranslated region[110].

However, certain characteristics of M2 polarized macrophages, do not conform to the straightforward distinction between pro- and anti-inflammatory properties that has been put forth by the M1/M2 paradigm. Firstly, M2 macrophages are highly phagocytic and express increased levels of scavenging receptors such as scavenger receptor class A (SR-A) and CD36. Both these receptors have originally been described as major mediators of foam cell formation and promoters of atherosclerosis in general. However, recent studies have questioned the function of these scavenger receptors as being mere facilitators of lipid uptake and even advocated them as mediators of inflammation in atherogenesis[111,112]. Accordingly, M2 polarization may very well promote foam cell formation and thereby lesion growth, but may also add to a lesion's inflammatory burden through changes in scavenger receptor activity. Secondly, as described above, through tumour biology research it is well known that M2 macrophages promote angiogenesis[113]. Especially the so-called angiogenic switch, which concerns the induction of tumour vasculature that is conditional for tumour growth and disease progression, is considered to be at least partly mediated by TAMs[113,114]. Hypothetically, M2 macrophages in the vessel wall may similarly promote microvessel growth into atherosclerotic lesions. However, as these newly formed vessels often lack proper structural integrity, they can give rise to intraplaque haemorrhage that in turn promotes lesion development and compromises plaque stability[115].

Hence, despite the fact that the cytokine and chemokine repertoires raise high hopes for M2 macrophages as therapeutic targets in atherosclerosis, not all functional characteristics exerted by these cells are by definition beneficial. Even so, it is important to recognize that any undesirable effects need not necessarily concern all M2 subtypes, since these factors might just as well be attributable to a single M2 phenotype. Selecting an appropriate subset that promotes the desired properties will therefore be crucial for the development of diagnostic and treatment options. In this view, characterizing the temporal and spatial balance between macrophage subsets and its underlying mechanisms provides an interesting challenge that might open doors towards new methods of atherosclerosis diagnosis and therapy, namely by targeting, favouring or inhibiting specific cell types and functions. That said, one of the major obstacles that will have to be overcome when studying macrophage polarization in atherosclerosis is the current lack of a good readout system that uses markers to specifically distinguish the different phenotypes. Despite the existence of many accounts listing typical genes, proteins and metabolic products associated with the different subpopulations, these markers are usually more abundant in, but rarely specific for, a particular subset. To be able to recognize the different subsets within one tissue, a more clear-cut readout system would be advantageous. Moreover, most of the M1 and M2 associated markers have not been tested for their expression and intralesional localization in either human or experimental atherosclerosis models. Hence, to better understand the functions exerted by the different subsets, their

characteristics should be examined further on a molecular and phenotypic level. In addition, since atherosclerotic plaques contain a unique macrophage stimulus in mLDL, it will be of great interest to study if this at all alters the response of macrophages to the typical M1 and M2 inducing signals. Still, lipid-laden foam cells could possibly be seen as yet another phenotype with characteristics distinct from the current M1 and M2 phenotypes. Finally, since different subsets are present in the plaque and probably co-exist for extended periods within one tissue, it would also be valuable to study the interplay between macrophages with different phenotypes. An important feature to investigate here is the ability of macrophages to redifferentiate into another phenotype in response to stimuli from their environment[10].

Macrophage polarizing cytokines in atherosclerosis

To actually study the role of the different macrophage subsets in atherogenesis, specific gene targeting in different murine models of atherosclerosis remains the method of choice. Up until now, a broad range of studies deleting or over-expressing cytokines with key functions in macrophage polarization have been performed in both $ApoE^{-/-}$ and $LDLR^{-/-}$ mice. However these investigations never focused directly on how a specific intervention affects macrophage phenotype in the plaque, but rather on atherosclerosis development in general[93].

In the setting of atherosclerosis, IL-10 is one of the best studied polarizing cytokines. As described earlier, IL-10 is involved in M2c polarization, but has also been shown in several studies to exert atheroprotective qualities, especially in early atherogenesis. Several knockout models for this cytokine describe a significant increase in lesion size and inflammation, which is accompanied by reduced plaque stability and an increased influx of T-cells[116,117]. In line with these findings, different transgenic models overexpressing IL-10 demonstrate a reduced lesion size[118-120]. However, since IL-10 also favours Th2 responses and has anti-inflammatory effects on most other cell types present in the atherosclerotic plaque, we are currently unable to appreciate exactly how much of its anti-atherogenic effects is mediated by macrophage phenotype skewing. For this reason, additional studies addressing IL-10 signaling specifically in macrophages should be performed that clearly dissect the inability of these macrophages to respond to or produce and secrete IL-10 from that of other immune cells. Moreover, such studies may provide novel insights into the expression of markers for macrophage subsets in atherosclerosis and their dependence on polarizing signals.

Another polarizing cytokine, IL-4, rather induces an M2a phenotype in macrophages. Although these are potentially anti-atherogenic, IL-4 is also known to be involved in the up-regulation of VCAM-1[121,122] and MCP-1[123,124] in endothelial cells, both important positive mediators of atherogenesis. This possibly blurs the effect of studies investigating the effects of IL-4 on atherosclerosis in mouse models and may be the reason why $ApoE^{-/-}IL\text{-}4^{-/-}$ double KO mice or $LDLR^{-/-}$ mice transplanted with $IL\text{-}4^{-/-}$ bone

marrow initially rather showed an atherosclerosis promoting effect in a time and site specific manner[125,126]. However, an initial study on IL-4 deficiency in C57BL/6 mice showed no effect[127] and more recently King et al. performed an extensive study with both ApoE$^{-/-}$ and LDLR$^{-/-}$ mice with IL-4 knockout or treatment approaches and also found no effect on atherosclerotic lesion formation[128]. These results raise questions concerning the contribution of M2a macrophages to atherogenesis, but may also be caused by the aforementioned opposing effects on different cellular populations in the plaques. Finally, contradicting evidence on IL-4 may even represent differences between mouse and man.

Comparable to the difficulties that arise in distinguishing the M2 and Th2 promoting capacities of IL-10, the effect of macrophage polarization by IFN-γ on atherogenesis is also difficult to reveal, since this pro-inflammatory cytokine affects both M1 macrophages and Th1 cells. Most experimental studies performed clearly show a reduction in lesion size upon absence of this cytokine or its receptor[129-131] and report accelerated atherosclerosis upon intraperitoneal recombinant IFN-γ administration[132]. These data confirm the atherogenic effect of this M1 polarizing cytokine. However, as is the case with IL-10, it is still unclear which cell types mediate the observed effects and whether genetic modification or treatment with IFN-γ actually altered macrophage populations and functionality in the plaques of these mice.

Last but not least, the pro-inflammatory TNF is probably one of the most difficult macrophage polarizing cytokines to study, due to its broad range of activities in atherosclerosis. Besides promoting an M1 phenotype, it also induces cell attraction through upregulation of intercellular adhesion molecule 1 (ICAM-1), VCAM-1 and MCP-1 in endothelial cells and influences foam cell formation through regulation of scavenger receptor mediated LDL uptake[133]. This abundance in TNF's possible interactions within several key stages of atherogenesis complicates the comparison of different experimental models, making it very difficult at this moment in time to distil a concrete theory on the effect of TNF-induced M1 macrophages on the atherosclerotic plaque. In general however, absence of TNF has been found to decrease atherosclerotic lesion size and progression[133-136].

In conclusion, the data above clearly shows association of polarizing cytokines with atherosclerosis development, as depicted in Figure 5.2 as well**.** However, studies on the direct effect of M1/M2 skewing on atherosclerosis in experimental models are lacking and more specifically, identification of subsets and their functional roles in vivo are scarce. To investigate this further, it may be important to realize that an approach of phenotypic switch should be preferred over the selective depletion of a specific subset. Targeting specific cell types with a cell death inducing method may lead to an accumulation of cell debris in the plaque, which causes plaque instability, especially in the more advanced lesions[137]. Another obstacle to keep in mind when further studying this matter is that both macrophage heterogeneity[138] and atherogenesis[139,140] are known to be differently regulated between species. Therefore, the major challenge in

the end will still be to translate novel findings from murine atherosclerosis models to the human situation.

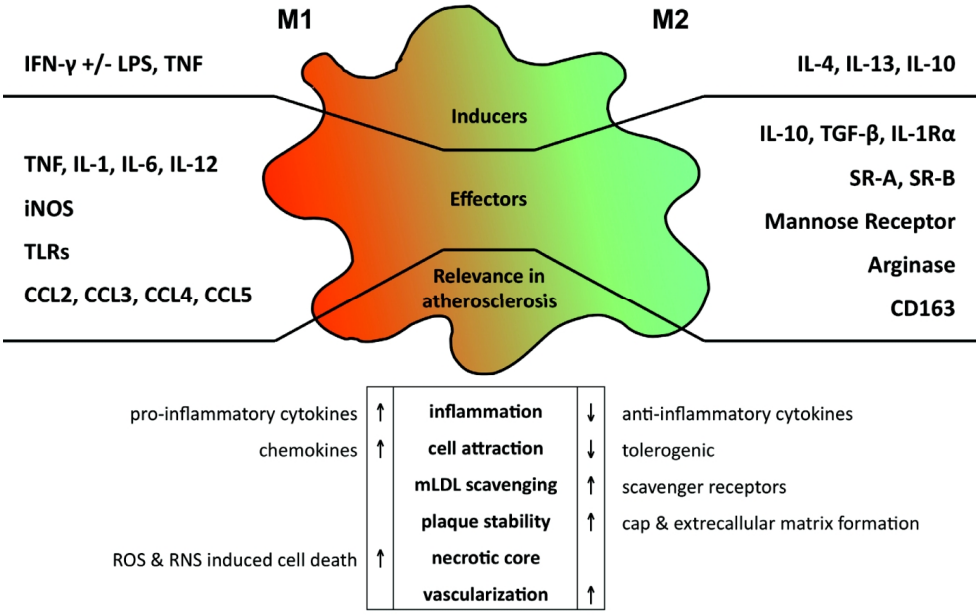

Figure 5.2: Macrophage polarization in atherosclerosis:

Summary of macrophage properties that are altered by phenotype skewing and are known to play a role in the atherosclerotic plaque. The top part of the figure shows a list of cytokines and other effectors that are typically associated with macrophage heterogeneity and have already been described to be relevant in terms of atherosclerosis development. Underneath (in bold) is a list of macrophage functions that are known to be involved in atherogenesis. The arrows indicate how macrophage polarization affects these processes. They are accompanied by the mechanism or effectors that are responsible for this altered function.

Macrophage polarization: beneficial effects of therapeutics and future opportunities for imaging?

Numerous studies provide an indication for therapeutic agents, of which the function can at least partly be ascribed to their immuno-modulatory properties. Hypothetically, distinct macrophage subsets might be involved in mediating the (pleiotropic) actions of some of these drugs. In turn, these agents might drive macrophage polarization to a certain extent as well and may thereby present ways to manipulate macrophage reprogramming. Although a multitude of therapeutics may be worth considering in this respect (e.g. type I interferons, corticosteroids), we opted to highlight just a selection of compounds that are readily prescribed in current cardiovascular medicine. Nevertheless, it would most certainly be interesting to pursue other drugs for their

possible influence on macrophage phenotypes and even explore potential synergies between different immune-modulators. In addition, imaging of macrophages as a diagnostic tool has recently gained great interest. Since macrophage subsets are likely to be differentially involved in regulating plaque instability, future approaches based on the identification of certain detrimental macrophage subsets (or associated characteristics) may be of prognostic value in atherosclerosis. We focus on a number of studies reporting macrophage-based strategies for atherosclerosis imaging and speculate on potential methods for discriminating between macrophage subpopulations.

Therapeutic interventions modulating macrophage polarization?

Statins (3-hydroxy-3-methylglutaryl coenzyme A or HMG-coA reductase inhibitors), provide a proficient approach to achieve lowering of serum cholesterol in patients and have accordingly become one of the most commonly used drugs in the primary and secondary prevention of cardiovascular disease[141]. Over the last decade, several in vitro and in vivo studies have established that statins conduct at least part of their beneficial effects through a manner that is independent of their lipid-lowering properties [142,143]. As a result, statins have gained strong interest in different fields of inflammatory disease, as several of their pleiotropic effects were demonstrated to be attributable to the modulation of inflammatory processes. First of all, these drugs have been demonstrated to inhibit inducible MHC-II expression by IFN-γ in monocyte-macrophages and endothelial cells[144]. Moreover, in 2002, Youssef et al. showed that the HMG-coA reductase inhibitor atorvastatin significantly alters the inflammatory balance in a murine model of auto-immune encephalomyelitis[145]. Whilst inhibiting secretion of the pro-atherogenic Th1-cytokines IL-12, IFN-γ and TNF, atorvastatin promoted a shift towards a Th2-type immune response through the release of cytokines such as IL-4, IL-5, IL-10 and TGF-β. Moreover, this study provides further confirmation that statins affect both T-cell and antigen-presenting cell (APC) compartments. Similarly, a decrease in Th1 bias was reported in human study populations[146,147], providing additional in vivo evidence for the immunoregulatory potential of statins. With regard to macrophage polarization, these findings may be of particular interest, since changing the immune response through treatment with statins might shift the phenotype of macrophage populations. Apart from being acknowledged as anti-atherogenic factors, Th2 effector molecules are evidently engrossed in the skewing of macrophages towards M2 phenotypes[13]. For this reason, statin-mediated alterations in inflammatory processes might well offer an interesting mechanism for shaping macrophage polarization. Admittedly, as a therapeutic class, statins are comprised of many individual agents that may have varying degrees of immuno-modulatory potential and thus may differently influence macrophage phenotype switching. However, to our knowledge, work on this subject is yet to be reported. Finally, rather than being limited to the development of atherosclerosis, differential

involvement of macrophage subsets may potentially be incorporated in other inflammatory disorders as well. As such, statins have been shown to exert anti-inflammatory effects in rheumatoid arthritis[148] and to inhibit the amount and size of brain lesions in multiple sclerosis (MS)[149].

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are PPARγ agonists that are used to increase insulin sensitivity in type 2 diabetes patients. In addition to their metabolic action, these compounds have also been shown to exhibit profound anti-atherosclerotic effects in several animal models[150-152]. These atheroprotective functions (e.g. reduced M1-cytokines and MMP-9 production[153,154]) are largely attributed to their immuno-modulatory properties, since PPARγ can directly interfere with inflammatory signal transduction cascades such as NF-κB, STAT-1 and activating protein-1[155]. In line with this anti-inflammatory action, rosiglitazone treatment has been shown to reduce plasma levels of C-reactive protein in type 2 diabetes patients[156]. As discussed above, it was shown that PPARγ agonists can directly drive macrophage polarization towards an M2 programme in both human and mouse cells[30,78]. Thus, an important part of the anti-atherosclerotic and perhaps even the anti-diabetic action of PPARγ activation may be attributed to polarization of macrophages to an M2 phenotype. In a recent randomized placebo-controlled trial in non-diabetic patients, rosiglitazone was indeed shown to reduce lesional inflammation and increased plaque collagen content and stability[157]. Interestingly, all of these effects are associated features of M2 macrophages. However, examination of macrophage gene expression identified certain M1 and M2-associated genes to be unresponsive to TZDs[79,158], implying that activation of PPARγ through agonists or ligands is influential, but not solely responsible for M2 polarization. More investigation into this area is needed to clarify the contribution of M1 and M2 macrophages and the role of PPARs in both atherosclerosis and diabetes.

The macrophage as a diagnostic target

As a large body of evidence has designated a lesion's inflammatory burden, rather than the degree of stenosis, as the major determining factor for clinical outcome in atherosclerosis[1,82], newly developed imaging strategies accordingly aim to assess the key inflammatory components that decide a plaque's fate. Seeing that polarized macrophage subsets are hypothesized to highly affect plaque stability, supposedly with contrasting outcomes, imaging strategies that target specific macrophage populations or the polarization balance in general might prove valuable tools in the prevention of acute ischemic events.

Above all, magnetic resonance imaging (MRI) is considered to be of great potential in terms of atherosclerosis imaging[159]. Due to its excellent soft-tissue contrast, this non-invasive imaging modality is recognized as being supremely adept for serially assessing atherosclerotic vessels[160]. Through application of MRI in experimental animal models

(i.e. rabbits and mice) and human research populations, adequate in and ex vivo imaging protocols for the assessment of atherosclerotic plaque composition have been defined[161-163]. By discriminating plaque constituents based on differences in magnetic relaxation properties, this technique is able to detect cellular elements, lipid core, fibrosis, and thrombus formation in atherosclerotic lesions with high sensitivity and specificity[164]. Interestingly, the use of contrast enhancement provides additional opportunities for depicting inflammatory mediators in atherosclerosis (e.g. macrophages). One such an approach utilizes ultrasmall superparamagnetic particles of iron oxide (USPIO) as a contrast agent for MRI. Once internalized by macrophages, these nanoparticles cause a focal loss of signal intensity on MRI and thus enable non-invasive localization of macrophages in vivo[165]. At present, this technique has been used to assess atherosclerotic plaques in hyperlipidemic rabbits[166,167], ApoE$^{-/-}$ mice[168] and was demonstrated to be a feasible strategy for macrophage detection in a human population[169]. Interestingly, the latter study reported accumulation of USPIO especially in macrophages in ruptured or rupture-prone atherosclerotic lesions. Hence, by appraising plaque macrophage content, USPIO-enhanced MRI might help distinguish between stable and vulnerable lesions.

In a similar fashion, several studies evaluated alternative imaging modalities for their ability to portray macrophages in atherosclerosis. Recently, Hyafil et al. demonstrated that the iodinated contrast agent N1177 is readily taken up by macrophages and subsequently induces an increased density of macrophage-rich tissues on computed tomography (CT) imaging[170]. Upon histological examination this effect co-localized with macrophage infiltration. In addition, Fayad and colleagues reported a CT-based approach to be suitable for plaque characterization[171]. Finally, positron emission tomography (PET) imaging techniques can visualize macrophages by detecting uptake of ^{18}F fluorodeoxyglucose (FDG). Macrophage-rich atherosclerotic lesions show augmented uptake of FDG due to the higher metabolic activity of activated macrophages. Interestingly, symptomatic lesions exhibit more FDG accumulation in comparison to their asymptomatic counterparts[172], further implicating macrophages in plaque complication.

Our growing insights into the molecular mechanisms that drive atherosclerosis emphasize the need for imaging techniques that are able to depict functional and biological processes (e.g. inflammation) as opposed to anatomical abnormalities (e.g. stenosis). Taken together, the accounts above use a myriad of ways to visualize macrophages in atherosclerotic lesions and thereby show these cells to be both promising and versatile targets for future imaging strategies. Although these methods need to be optimized before routine application in clinical practice can be realized, they may ultimately extend to the depiction of macrophage subsets in inflammatory disease. Striving to further unravel the involvement of distinct macrophage subsets in atherogenesis will surely propagate the development of novel imaging strategies that grant us information regarding the macrophage polarization balance in selected parts

of the vasculature. Attaining such data prior to the patient developing symptoms might revolutionize the ways by which we assess atherosclerotic arteries, stratify individual risk and might even allow us to intervene accordingly. For instance, selectively visualizing a lesion's M1 macrophage content could point out plaques containing a relatively high inflammatory load that as such have a higher likelihood to experience rapid progression or even rupture. The role of different M2 macrophage subsets in atherosclerosis however is still somewhat controversial. As we mentioned before, specific features that are possibly attributable to a M2 subset (i.e. angiogenesis) may actually enhance atherogenesis, leaving that particular phenotype less desirable in atherosclerosis. Conversely, we certainly expect other M2 phenotypes to be beneficial for plaque stability. For this reason, reflecting on how imaging of M2 macrophages would likely be implemented in the diagnostic work-up of atherosclerosis is difficult. To illustrate this, we would like to refer to an earlier section of this review, in which we elaborately discuss that an M2 population of $CD163^{high}$ macrophages in atherosclerotic lesions could identify plaques that are predisposed to rupture through intraplaque haemorrhage. This particular example creates a remarkable controversy that shows the complexity of the M1/M2 paradigm, since an atheroprotective M2 subset may possibly also serve as a diagnostic tool for recognizing certain detrimental plaque characteristics. Therefore, depending on the M2 subset in question, a visual appraisal of lesional M2 content might provide additional, but diverging clues as to a plaque's eventual outcome.

However, at this time we can only speculate on possible diagnostic techniques that will allow for a dynamic application of macrophage heterogeneity in atherosclerosis. Ideally, continued efforts to characterize macrophage subsets will identify better (e.g. subset- but not species-specific) markers that could then be specifically targeted for imaging. In addition, differential affinity of polarized macrophage subtypes for the internalization of certain molecules and compounds might offer another window of opportunity for subset visualization.

Conclusions

Over the past few years, we gained considerable insight into the inflammatory processes that lie at the core of atherosclerosis development. Macrophages are progressively acknowledged as major determinants of atherosclerosis, joining both innate and adaptive immune responses in its pathogenesis. Currently, several polarized M1 and M2 macrophage subsets have been identified and both populations were demonstrated to be present in atherosclerotic lesions. Although the differential expression of effector phenotype implies macrophage function in atherosclerosis to be highly ambiguous (i.e. M1 and M2 macrophages as atherogenic and atheroprotective effectors respectively), evidence supporting their functional implications in vivo is

largely lacking. In this respect, several arising challenges complicate the implementation of macrophage polarization in our understanding of the atherosclerotic process. Firstly, additional markers are required that will allow us to better distinguish the current macrophage subsets and possibly help identify new ones. Preferably, these markers will be adept for characterizing both murine and human macrophage populations, as inter-species variability is becoming increasingly apparent. Any such markers might subsequently facilitate the exploration of means by which the inflammatory balance in atherogenesis can be affected (e.g. by pharmaceutical intervention). Additionally, we need direct functional studies in mice that lack a specific macrophage subset to clarify the in vivo relevance of that particular subtype and possibly increase our insight into the underlying mechanisms.

Ultimately, the current M1/M2 paradigm offers a comprehensive take on the concept of macrophage heterogeneity. However, due to its simplified nature, it is highly unlikely that this digotomy represents the full scope of functional macrophage phenotypes. Instead, the current M1 and M2 states are better regarded as extremes of a continuum that remains to be explored. Hence, if we are to generate a classification that is a more accurate portrayal of in vivo atherosclerotic conditions, we should advance our knowledge on this phenomenon. Only by negotiating the aforementioned difficulties will we be able to develop strategies that allow us to skew macrophage polarization in such a manner that it serves our diagnostic and therapeutic goals, be it in the context of atherosclerosis or other macrophage-mediated disorders such as obesity and cancer.

References

1. Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868-874 (2002).
2. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).
3. Geissmann, F., Jung, S. & Littman, D.R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71-82 (2003).
4. Mantovani, A.*, et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **25**, 677-686 (2004).
5. Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* **3**, 23-35 (2003).
6. Gordon, S. & Taylor, P.R. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**, 953-964 (2005).
7. Helming, L. & Gordon, S. Macrophage fusion induced by IL-4 alternative activation is a multistage process involving multiple target molecules. *Eur J Immunol* **37**, 33-42 (2007).
8. Van Ginderachter, J.A.*, et al.* Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology* **211**, 487-501 (2006).
9. Stout, R.D. & Suttles, J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol* **76**, 509-513 (2004).
10. Porcheray, F.*, et al.* Macrophage activation switching: an asset for the resolution of inflammation. *Clin Exp Immunol* **142**, 481-489 (2005).
11. Stout, R.D.*, et al.* Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* **175**, 342-349 (2005).
12. Lawrence, T. & Gilroy, D.W. Chronic inflammation: a failure of resolution? *Int J Exp Pathol* **88**, 85-94 (2007).
13. Martinez, F.O., Sica, A., Mantovani, A. & Locati, M. Macrophage activation and polarization. *Front Biosci* **13**, 453-461 (2008).
14. Mosser, D.M. & Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8**, 958-969 (2008).
15. Mantovani, A., Sica, A. & Locati, M. Macrophage polarization comes of age. *Immunity* **23**, 344-346 (2005).
16. Goerdt, S.*, et al.* Alternative versus classical activation of macrophages. *Pathobiology* **67**, 222-226 (1999).
17. Martinez, F.O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* **177**, 7303-7311 (2006).
18. Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J. & Hill, A.M. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* **164**, 6166-6173 (2000).
19. Mosser, D.M. The many faces of macrophage activation. *J Leukoc Biol* **73**, 209-212 (2003).
20. Mukhopadhyay, S.*, et al.* MARCO, an innate activation marker of macrophages, is a class A scavenger receptor for Neisseria meningitidis. *Eur J Immunol* **36**, 940-949 (2006).
21. Skeen, M.J., Miller, M.A., Shinnick, T.M. & Ziegler, H.K. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. *J Immunol* **156**, 1196-1206 (1996).
22. Martinez, F.O., Helming, L. & Gordon, S. Alternative Activation of Macrophages: An Immunologic Functional Perspective. *Annu Rev Immunol* (2008).

23. Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* **176**, 287-292 (1992).
24. Anderson, C.F. & Mosser, D.M. A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J Leukoc Biol* **72**, 101-106 (2002).
25. Anderson, C.F. & Mosser, D.M. Cutting edge: biasing immune responses by directing antigen to macrophage Fc gamma receptors. *J Immunol* **168**, 3697-3701 (2002).
26. Edwards, J.P., Zhang, X., Frauwirth, K.A. & Mosser, D.M. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol* **80**, 1298-1307 (2006).
27. Mantovani, A., Sica, A. & Locati, M. New vistas on macrophage differentiation and activation. *Eur J Immunol* **37**, 14-16 (2007).
28. Hesse, M.*, et al.* Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* **167**, 6533-6544 (2001).
29. Waldo, S.W.*, et al.* Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *Am J Pathol* **172**, 1112-1126 (2008).
30. Bouhlel, M.A.*, et al.* PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* **6**, 137-143 (2007).
31. Ghassabeh, G.H.*, et al.* Identification of a common gene signature for type II cytokine-associated myeloid cells elicited in vivo in different pathologic conditions. *Blood* **108**, 575-583 (2006).
32. Jenner, R.G. & Young, R.A. Insights into host responses against pathogens from transcriptional profiling. *Nat Rev Microbiol* **3**, 281-294 (2005).
33. Nau, G.J.*, et al.* Human macrophage activation programs induced by bacterial pathogens. *Proc Natl Acad Sci U S A* **99**, 1503-1508 (2002).
34. Benoit, M., Desnues, B. & Mege, J.L. Macrophage polarization in bacterial infections. *J Immunol* **181**, 3733-3739 (2008).
35. Bozza, F.A.*, et al.* Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. *Crit Care* **11**, R49 (2007).
36. Benoit, M., Barbarat, B., Bernard, A., Olive, D. & Mege, J.L. Coxiella burnetii, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur J Immunol* **38**, 1065-1070 (2008).
37. Benoit, M., Ghigo, E., Capo, C., Raoult, D. & Mege, J.L. The uptake of apoptotic cells drives Coxiella burnetii replication and macrophage polarization: a model for Q fever endocarditis. *PLoS Pathog* **4**, e1000066 (2008).
38. Meghari, S.*, et al.* Persistent Coxiella burnetii infection in mice overexpressing IL-10: an efficient model for chronic Q fever pathogenesis. *PLoS Pathog* **4**, e23 (2008).
39. Desnues, B., Lepidi, H., Raoult, D. & Mege, J.L. Whipple disease: intestinal infiltrating cells exhibit a transcriptional pattern of M2/alternatively activated macrophages. *J Infect Dis* **192**, 1642-1646 (2005).
40. Noel, W., Raes, G., Hassanzadeh Ghassabeh, G., De Baetselier, P. & Beschin, A. Alternatively activated macrophages during parasite infections. *Trends Parasitol* **20**, 126-133 (2004).
41. Borst, P. & Rudenko, G. Antigenic variation in African trypanosomes. *Science* **264**, 1872-1873 (1994).
42. Namangala, B., De Baetselier, P., Noel, W., Brys, L. & Beschin, A. Alternative versus classical macrophage activation during experimental African trypanosomosis. *J Leukoc Biol* **69**, 387-396 (2001).

43. Baetselier, P.D.*, et al.* Alternative versus classical macrophage activation during experimental African trypanosomosis. *Int J Parasitol* **31**, 575-587 (2001).
44. Bosschaerts, T.*, et al.* Alternatively activated myeloid cells limit pathogenicity associated with African trypanosomiasis through the IL-10 inducible gene selenoprotein P. *J Immunol* **180**, 6168-6175 (2008).
45. Magez, S., Radwanska, M., Beschin, A., Sekikawa, K. & De Baetselier, P. Tumor necrosis factor alpha is a key mediator in the regulation of experimental Trypanosoma brucei infections. *Infect Immun* **67**, 3128-3132 (1999).
46. Shi, M., Pan, W. & Tabel, H. Experimental African trypanosomiasis: IFN-gamma mediates early mortality. *Eur J Immunol* **33**, 108-118 (2003).
47. Namangala, B., Noel, W., De Baetselier, P., Brys, L. & Beschin, A. Relative contribution of interferon-gamma and interleukin-10 to resistance to murine African trypanosomosis. *J Infect Dis* **183**, 1794-1800 (2001).
48. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **23**, 549-555 (2002).
49. Smyth, M.J., Godfrey, D.I. & Trapani, J.A. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* **2**, 293-299 (2001).
50. Bronte, V. & Zanovello, P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* **5**, 641-654 (2005).
51. Pollard, J.W. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* **4**, 71-78 (2004).
52. Mantovani, A., Schioppa, T., Porta, C., Allavena, P. & Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev* **25**, 315-322 (2006).
53. Bottazzi, B.*, et al.* Regulation of the macrophage content of neoplasms by chemoattractants. *Science* **220**, 210-212 (1983).
54. Matsushima, K., Larsen, C.G., DuBois, G.C. & Oppenheim, J.J. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J Exp Med* **169**, 1485-1490 (1989).
55. Bonnotte, B.*, et al.* Identification of tumor-infiltrating macrophages as the killers of tumor cells after immunization in a rat model system. *J Immunol* **167**, 5077-5083 (2001).
56. Mytar, B.*, et al.* Induction of reactive oxygen intermediates in human monocytes by tumour cells and their role in spontaneous monocyte cytotoxicity. *Br J Cancer* **79**, 737-743 (1999).
57. de Visser, K.E., Eichten, A. & Coussens, L.M. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6**, 24-37 (2006).
58. Weigert, A.*, et al.* Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. *Mol Biol Cell* **18**, 3810-3819 (2007).
59. Lewis, C.E. & Pollard, J.W. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66**, 605-612 (2006).
60. Burke, B.*, et al.* Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. *J Pathol* **196**, 204-212 (2002).
61. Biswas, S.K.*, et al.* A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood* **107**, 2112-2122 (2006).

62. Sica, A.*, et al.* Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. *J Immunol* **164**, 762-767 (2000).
63. Bonizzi, G. & Karin, M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**, 280-288 (2004).
64. Saccani, A.*, et al.* p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res* **66**, 11432-11440 (2006).
65. Fong, C.H.*, et al.* An antiinflammatory role for IKKbeta through the inhibition of "classical" macrophage activation. *J Exp Med* **205**, 1269-1276 (2008).
66. Hagemann, T.*, et al.* "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *J Exp Med* **205**, 1261-1268 (2008).
67. Bingle, L., Brown, N.J. & Lewis, C.E. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* **196**, 254-265 (2002).
68. Condeelis, J. & Pollard, J.W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-266 (2006).
69. Lin, E.Y., Nguyen, A.V., Russell, R.G. & Pollard, J.W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* **193**, 727-740 (2001).
70. Nowicki, A.*, et al.* Impaired tumor growth in colony-stimulating factor 1 (CSF-1)-deficient, macrophage-deficient op/op mouse: evidence for a role of CSF-1-dependent macrophages in formation of tumor stroma. *Int J Cancer* **65**, 112-119 (1996).
71. Robinson, S.C.*, et al.* A chemokine receptor antagonist inhibits experimental breast tumor growth. *Cancer Res* **63**, 8360-8365 (2003).
72. Shoelson, S.E., Lee, J. & Goldfine, A.B. Inflammation and insulin resistance. *J Clin Invest* **116**, 1793-1801 (2006).
73. Harman-Boehm, I.*, et al.* Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab* **92**, 2240-2247 (2007).
74. Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175-184 (2007).
75. Lumeng, C.N., DelProposto, J.B., Westcott, D.J. & Saltiel, A.R. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* **57**, 3239-3246 (2008).
76. Kanda, H.*, et al.* MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* **116**, 1494-1505 (2006).
77. Weisberg, S.P.*, et al.* CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* **116**, 115-124 (2006).
78. Odegaard, J.I.*, et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* **447**, 1116-1120 (2007).
79. Hevener, A.L.*, et al.* Macrophage PPAR gamma is required for normal skeletal muscle and hepatic insulin sensitivity and full antidiabetic effects of thiazolidinediones. *J Clin Invest* **117**, 1658-1669 (2007).
80. Odegaard, J.I.*, et al.* Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab* **7**, 496-507 (2008).
81. Kang, K.*, et al.* Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell Metab* **7**, 485-495 (2008).
82. Lusis, A.J. Atherosclerosis. *Nature* **407**, 233-241 (2000).
83. Glass, C.K. & Witztum, J.L. Atherosclerosis. the road ahead. *Cell* **104**, 503-516 (2001).

84. Peiser, L., Mukhopadhyay, S. & Gordon, S. Scavenger receptors in innate immunity. *Curr Opin Immunol* **14**, 123-128 (2002).
85. Ross, R. Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**, 115-126 (1999).
86. Duffield, J.S. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)* **104**, 27-38 (2003).
87. van der Wal, A.C., Das, P.K., Tigges, A.J. & Becker, A.E. Macrophage differentiation in atherosclerosis. An in situ immunohistochemical analysis in humans. *The American journal of pathology* **141**, 161-168 (1992).
88. Gijbels, M.J*., et al.* Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* **143**, 15-25 (1999).
89. Boyle, J.J*., et al.* Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. *Am J Pathol* **174**, 1097-1108 (2009).
90. Papaspyridonos, M*., et al.* Novel candidate genes in unstable areas of human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* **26**, 1837-1844 (2006).
91. Schaer, D.J., Boretti, F.S., Schoedon, G. & Schaffner, A. Induction of the CD163-dependent haemoglobin uptake by macrophages as a novel anti-inflammatory action of glucocorticoids. *Br J Haematol* **119**, 239-243 (2002).
92. Kleemann, R., Zadelaar, S. & Kooistra, T. Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovasc Res* **79**, 360-376 (2008).
93. Tedgui, A. & Mallat, Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* **86**, 515-581 (2006).
94. Ohmori, Y. & Hamilton, T.A. Requirement for STAT1 in LPS-induced gene expression in macrophages. *J Leukoc Biol* **69**, 598-604 (2001).
95. Richmond, A. Nf-kappa B, chemokine gene transcription and tumour growth. *Nat Rev Immunol* **2**, 664-674 (2002).
96. Zernecke, A., Shagdarsuren, E. & Weber, C. Chemokines in atherosclerosis: an update. *Arterioscler Thromb Vasc Biol* **28**, 1897-1908 (2008).
97. Veillard, N.R*., et al.* Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* **94**, 253-261 (2004).
98. Kuziel, W.A*., et al.* CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. *Atherosclerosis* **167**, 25-32 (2003).
99. Potteaux, S*., et al.* Role of bone marrow-derived CC-chemokine receptor 5 in the development of atherosclerosis of low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* **26**, 1858-1863 (2006).
100. Quinones, M.P*., et al.* CC chemokine receptor 5 influences late-stage atherosclerosis. *Atherosclerosis* **195**, e92-103 (2007).
101. Braunersreuther, V*., et al.* Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* **27**, 373-379 (2007).
102. Potteaux, S*., et al.* Chemokine receptor CCR1 disruption in bone marrow cells enhances atherosclerotic lesion development and inflammation in mice. *Mol Med* **11**, 16-20 (2005).
103. Boring, L., Gosling, J., Cleary, M. & Charo, I.F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**, 894-897 (1998).
104. Gosling, J*., et al.* MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* **103**, 773-778 (1999).
105. Gu, L*., et al.* Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* **2**, 275-281 (1998).
106. Tacke, F*., et al.* Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest* **117**, 185-194 (2007).

107. Bonecchi, R.*, et al.* Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* **92**, 2668-2671 (1998).
108. Watanabe, K., Jose, P.J. & Rankin, S.M. Eotaxin-2 generation is differentially regulated by lipopolysaccharide and IL-4 in monocytes and macrophages. *J Immunol* **168**, 1911-1918 (2002).
109. Politz, O., Kodelja, V., Guillot, P., Orfanos, C.E. & Goerdt, S. Pseudoexons and regulatory elements in the genomic sequence of the beta-chemokine, alternative macrophage activation-associated CC-chemokine (AMAC)-1. *Cytokine* **12**, 120-126 (2000).
110. Teupser, D.*, et al.* Identification of macrophage arginase I as a new candidate gene of atherosclerosis resistance. *Arterioscler Thromb Vasc Biol* **26**, 365-371 (2006).
111. Manning-Tobin, J.J.*, et al.* Loss of SR-A and CD36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. *Arterioscler Thromb Vasc Biol* **29**, 19-26 (2009).
112. Moore, K.J. & Freeman, M.W. Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler Thromb Vasc Biol* **26**, 1702-1711 (2006).
113. Sica, A.*, et al.* Macrophage polarization in tumour progression. *Semin Cancer Biol* **18**, 349-355 (2008).
114. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-410 (2003).
115. Sluimer, J.C. & Daemen, M.J. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J Pathol* **218**, 7-29 (2009).
116. Caligiuri, G.*, et al.* Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med* **9**, 10-17 (2003).
117. Mallat, Z.*, et al.* Protective role of interleukin-10 in atherosclerosis. *Circ Res* **85**, e17-24 (1999).
118. Namiki, M.*, et al.* Intramuscular gene transfer of interleukin-10 cDNA reduces atherosclerosis in apolipoprotein E-knockout mice. *Atherosclerosis* **172**, 21-29 (2004).
119. Pinderski, L.J.*, et al.* Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. *Circ Res* **90**, 1064-1071 (2002).
120. Von Der Thusen, J.H.*, et al.* Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr-/- mice. *FASEB J* **15**, 2730-2732 (2001).
121. Lee, Y.W., Kuhn, H., Hennig, B., Neish, A.S. & Toborek, M. IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. *J Mol Cell Cardiol* **33**, 83-94 (2001).
122. Luscinskas, F.W.*, et al.* Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, beta 1-integrins, and beta 2-integrins. *J Cell Biol* **125**, 1417-1427 (1994).
123. Lee, Y.W., Hennig, B. & Toborek, M. Redox-regulated mechanisms of IL-4-induced MCP-1 expression in human vascular endothelial cells. *Am J Physiol Heart Circ Physiol* **284**, H185-192 (2003).
124. Rollins, B.J. & Pober, J.S. Interleukin-4 induces the synthesis and secretion of MCP-1/JE by human endothelial cells. *Am J Pathol* **138**, 1315-1319 (1991).
125. Davenport, P. & Tipping, P.G. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Pathol* **163**, 1117-1125 (2003).

126. King, V.L., Szilvassy, S.J. & Daugherty, A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. *Arterioscler Thromb Vasc Biol* **22**, 456-461 (2002).
127. George, J*., et al.* Interleukin (IL)-4 deficiency does not influence fatty streak formation in C57BL/6 mice. *Atherosclerosis* **153**, 403-411 (2000).
128. King, V.L., Cassis, L.A. & Daugherty, A. Interleukin-4 does not influence development of hypercholesterolemia or angiotensin II-induced atherosclerotic lesions in mice. *Am J Pathol* **171**, 2040-2047 (2007).
129. Buono, C*., et al.* Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse. *Arterioscler Thromb Vasc Biol* **23**, 454-460 (2003).
130. Gupta, S*., et al.* IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest* **99**, 2752-2761 (1997).
131. Whitman, S.C., Ravisankar, P. & Daugherty, A. IFN-gamma deficiency exerts gender-specific effects on atherogenesis in apolipoprotein E-/- mice. *J Interferon Cytokine Res* **22**, 661-670 (2002).
132. Whitman, S.C., Ravisankar, P., Elam, H. & Daugherty, A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. *Am J Pathol* **157**, 1819-1824 (2000).
133. Ohta, H*., et al.* Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis* **180**, 11-17 (2005).
134. Boesten, L.S*., et al.* Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. *Cardiovasc Res* **66**, 179-185 (2005).
135. Branen, L*., et al.* Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* **24**, 2137-2142 (2004).
136. Canault, M*., et al.* Exclusive expression of transmembrane TNF-alpha in mice reduces the inflammatory response in early lipid lesions of aortic sinus. *Atherosclerosis* **172**, 211-218 (2004).
137. Seimon, T. & Tabas, I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res* **50 Suppl**, S382-387 (2009).
138. Raes, G*., et al.* Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells. *J Immunol* **174**, 6561; author reply 6561-6562 (2005).
139. Zadelaar, S*., et al.* Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol* **27**, 1706-1721 (2007).
140. Smith, J.D. & Breslow, J.L. The emergence of mouse models of atherosclerosis and their relevance to clinical research. *J Intern Med* **242**, 99-109 (1997).
141. Maron, D.J., Fazio, S. & Linton, M.F. Current perspectives on statins. *Circulation* **101**, 207-213 (2000).
142. Arnaud, C., Braunersreuther, V. & Mach, F. Toward immunomodulatory and anti-inflammatory properties of statins. *Trends Cardiovasc Med* **15**, 202-206 (2005).
143. Ridker, P.M*., et al.* C-reactive protein levels and outcomes after statin therapy. *N Engl J Med* **352**, 20-28 (2005).
144. Kwak, B., Mulhaupt, F., Myit, S. & Mach, F. Statins as a newly recognized type of immunomodulator. *Nat Med* **6**, 1399-1402 (2000).
145. Youssef, S*., et al.* The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* **420**, 78-84 (2002).

146. Rosenson, R.S., Tangney, C.C. & Casey, L.C. Inhibition of proinflammatory cytokine production by pravastatin. *Lancet* **353**, 983-984 (1999).
147. Shimada, K., Miyauchi, K. & Daida, H. Early intervention with atorvastatin modulates TH1/TH2 imbalance in patients with acute coronary syndrome: from bedside to bench. *Circulation* **109**, e213-214; author reply e213-214 (2004).
148. McCarey, D.W.*, et al.* Trial of Atorvastatin in Rheumatoid Arthritis (TARA): double-blind, randomised placebo-controlled trial. *Lancet* **363**, 2015-2021 (2004).
149. Vollmer, T.*, et al.* Oral simvastatin treatment in relapsing-remitting multiple sclerosis. *Lancet* **363**, 1607-1608 (2004).
150. Chen, Z.*, et al.* Troglitazone inhibits atherosclerosis in apolipoprotein E-knockout mice: pleiotropic effects on CD36 expression and HDL. *Arterioscler Thromb Vasc Biol* **21**, 372-377 (2001).
151. Collins, A.R.*, et al.* Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* **21**, 365-371 (2001).
152. Li, A.C.*, et al.* Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* **106**, 523-531 (2000).
153. Jiang, C., Ting, A.T. & Seed, B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* **391**, 82-86 (1998).
154. Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J. & Glass, C.K. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* **391**, 79-82 (1998).
155. Chinetti, G., Fruchart, J.C. & Staels, B. Peroxisome proliferator-activated receptors: new targets for the pharmacological modulation of macrophage gene expression and function. *Curr Opin Lipidol* **14**, 459-468 (2003).
156. Haffner, S.M.*, et al.* Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. *Circulation* **106**, 679-684 (2002).
157. Meisner, F.*, et al.* Effect of rosiglitazone treatment on plaque inflammation and collagen content in nondiabetic patients: data from a randomized placebo-controlled trial. *Arterioscler Thromb Vasc Biol* **26**, 845-850 (2006).
158. Chawla, A.*, et al.* PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med* **7**, 48-52 (2001).
159. Leiner, T.*, et al.* Magnetic resonance imaging of atherosclerosis. *Eur Radiol* **15**, 1087-1099 (2005).
160. Fayad, Z.A. & Fuster, V. Characterization of atherosclerotic plaques by magnetic resonance imaging. *Ann N Y Acad Sci* **902**, 173-186 (2000).
161. Skinner, M.P.*, et al.* Serial magnetic resonance imaging of experimental atherosclerosis detects lesion fine structure, progression and complications in vivo. *Nat Med* **1**, 69-73 (1995).
162. Fayad, Z.A.*, et al.* Noninvasive In vivo high-resolution magnetic resonance imaging of atherosclerotic lesions in genetically engineered mice. *Circulation* **98**, 1541-1547 (1998).
163. Toussaint, J.F., LaMuraglia, G.M., Southern, J.F., Fuster, V. & Kantor, H.L. Magnetic resonance images lipid, fibrous, calcified, hemorrhagic, and thrombotic components of human atherosclerosis in vivo. *Circulation* **94**, 932-938 (1996).
164. Shinnar, M.*, et al.* The diagnostic accuracy of ex vivo MRI for human atherosclerotic plaque characterization. *Arterioscler Thromb Vasc Biol* **19**, 2756-2761 (1999).

165. Schmitz, S.A.*, et al.* Magnetic resonance imaging of atherosclerotic plaques using superparamagnetic iron oxide particles. *J Magn Reson Imaging* **14**, 355-361 (2001).
166. Ruehm, S.G., Corot, C., Vogt, P., Kolb, S. & Debatin, J.F. Magnetic resonance imaging of atherosclerotic plaque with ultrasmall superparamagnetic particles of iron oxide in hyperlipidemic rabbits. *Circulation* **103**, 415-422 (2001).
167. Schmitz, S.A.*, et al.* Superparamagnetic iron oxide-enhanced MRI of atherosclerotic plaques in Watanabe hereditable hyperlipidemic rabbits. *Invest Radiol* **35**, 460-471 (2000).
168. Klug, K.*, et al.* Murine atherosclerotic plaque imaging with the USPIO Ferumoxtran-10. *Front Biosci* **14**, 2546-2552 (2009).
169. Kooi, M.E.*, et al.* Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by in vivo magnetic resonance imaging. *Circulation* **107**, 2453-2458 (2003).
170. Hyafil, F.*, et al.* Noninvasive detection of macrophages using a nanoparticulate contrast agent for computed tomography. *Nat Med* **13**, 636-641 (2007).
171. Fayad, Z.A., Fuster, V., Nikolaou, K. & Becker, C. Computed tomography and magnetic resonance imaging for noninvasive coronary angiography and plaque imaging: current and potential future concepts. *Circulation* **106**, 2026-2034 (2002).
172. Rudd, J.H.*, et al.* Imaging atherosclerotic plaque inflammation with [18F]-fluorodeoxyglucose positron emission tomography. *Circulation* **105**, 2708-2711 (2002).

Chapter 6

General discussion

Through its versatility, the macrophage is a key player in both innate and adaptive immune responses. Its broad range of functions is illustrated at its best in infectious diseases, where they participate in pathogen killing and engulfment, attract other leukocytes to the site of infection, and ultimately help in directing the response of these newly recruited cells[1]. But also the outcome of non-infectious diseases like tumorigenesis[2] and insulin resistance[3] depends on the inflammatory regulation and phagocytic capacity of the macrophage.

In atherosclerosis, the multi-functionality of the macrophages is reflected in their different actions throughout the course of the disease[4]. After they initiate atherogenesis by taking up modified lipids in the vessel wall to a point where they are unable to leave the subendothelial space, they create a local inflammatory environment by producing and secreting chemokines, cytokines and growth factors. Hereby, they promote the attraction, activation and proliferation of new macrophages and other cells to the growing lesion. Their subsequent contribution to different processes can be either plaque stabilizing or destabilizing, a heterogeneity that is described in chapter 5.

Atherosclerosis and its acute clinical complications are the number one cause for mortality in Western society. Although not a newly emerging disease, the combination of a sedentary life style, high-caloric diet pattern and several environmental factors has increased our morbidity and mortality to alarmingly high levels. Through our knowledge of risk factors contributing to this disease, a number of preventive, lifestyle changing actions can be advised in order to reduce the risk. However, our advancing understanding of the molecular and cellular mechanisms underlying the pathogenesis has yet resulted in a disappointingly limited array of pharmaceutical or therapeutical options. In addition to tackling the hyperlipidemia that is caused through lifestyle or genetic predisposition[5] and surgically correcting the life-threatening clinical manifestations[6], therapeutics that can alter the course of the disease would be more than welcome.

Experiments in animal models have illustrated several strategies to interfere with molecular pathways and cellular interactions involved in atherogenesis that may present useful future treatment strategies but are still not readily applicable in the clinic. Because it is the most abundant cell type during plaque growth, the target of these therapies is often the macrophage and the several ways it contributes to the pathogenesis, from recruitment over foam cell formation to cell death and necrotic core formation. In this thesis, we further investigated the role of macrophages in atherogenesis, focusing on their recruitment to the plaque and inflammatory signaling pathways.

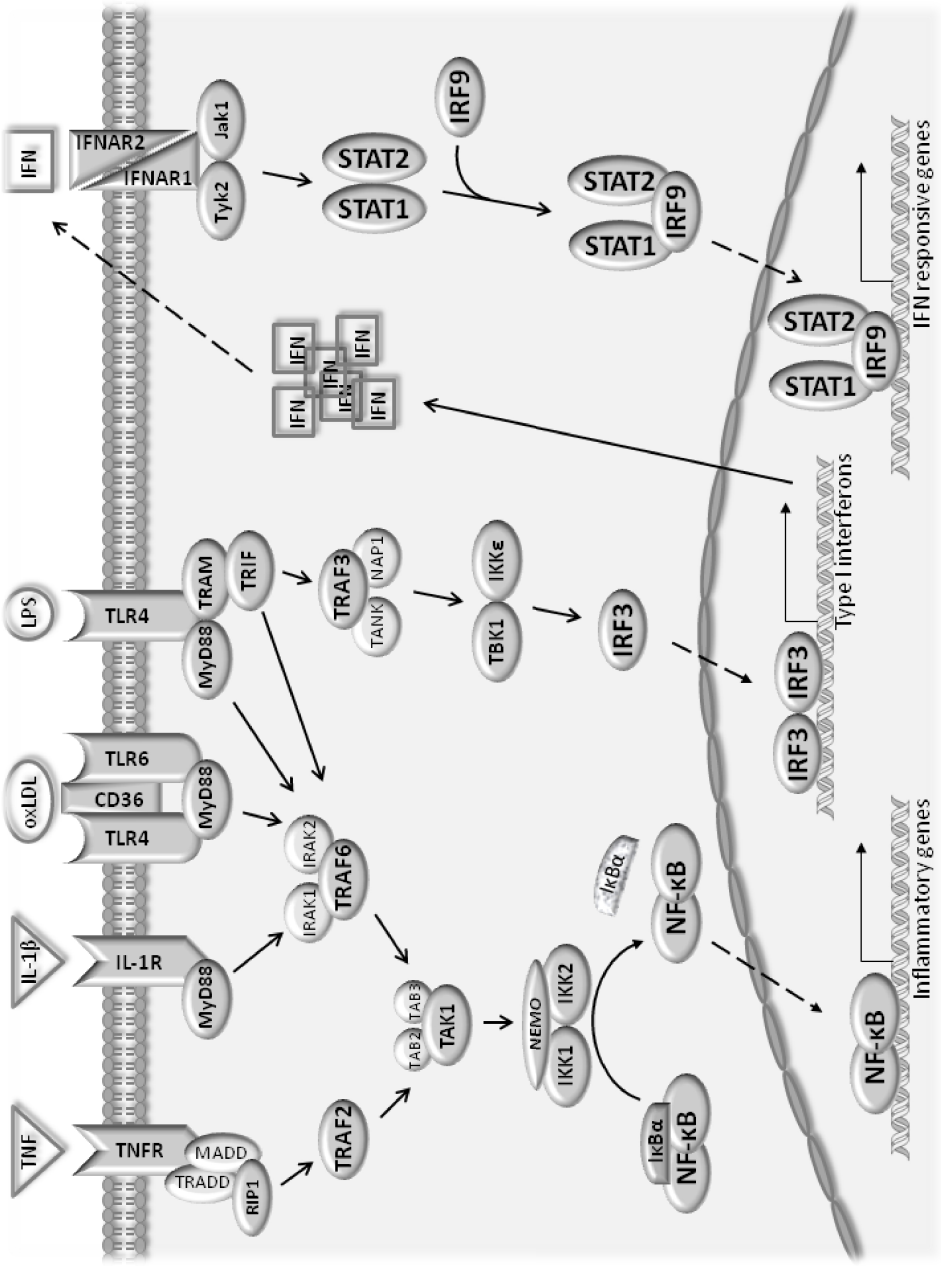

Figure 6.1: *The NF-κB and IRF3 signaling pathways that lead to the expression of NF-κB dependent genes and type I interferons, followed by type I interferon signaling.*

Type I interferons promote atherosclerosis

In **Chapter 2**, the effect of type I interferon (IFNα and IFNβ) treatment as well as the role of endogenous interferons on different mouse models of atherosclerosis was investigated.

Interferons were described more than 50 years ago as a cytokine family that is produced and secreted in response to virus infection and induces an antiviral state in the surrounding cells[7]. Exposure to specific viral or bacterial components activates Toll-like receptor signaling which then leads to interferon production, parallel to the activation of the inflammation-regulating transcription factor NF-κB (Figure 6.1). Upon secretion, autocrine or paracrine binding of interferons to their heterodimeric receptor (IFNAR1 and IFNAR2) will induce a Jak/STAT pathway that ensures the expression of a set of interferon-stimulated genes (ISGs)[8]. This diverse group of more than 300 genes regulates the cell's defense against microbial threats, with multiple redundant actions including active elimination of pathogens, raising the cell's resistance to infection and slowing down the protein production in order to halt the *de novo* assembly of parasites[9-11]. Additional to these antiviral properties however, the ISGs were also found to be regulating other cell functions that can play a role in case of infection. They can facilitate cell death, presumably to kill infected cells before they can contribute to the microbial expansion, and they have immune-regulatory functions, simultaneously restricting a potentially exorbitant innate immune response to the infection and encouraging an adaptive immune response.

Already since the early '80s, the therapeutic potency of these different type I interferon features is exploited in the clinic for the treatment of several diseases[8], with side effects that are mainly limited to flu-like symptoms and injection site irritation[12]. While patients infected with certain viruses, e.g. hepatitis C virus, benefit from the antiviral effects of interferons[13], some cancer patients have increased tumor cell necrosis upon interferon injections[14,15]. A more prolonged treatment is applied in patients suffering from relapsing-remitting multiple sclerosis (MS), a T cell-driven, chronic inflammatory disease that is characterized by episodic attacks of progressive neurological deterioration. Daily to weekly injections with interferons extends the period between the acute attacks through a yet largely unclear mechanism[16]. Both in these patients as well as in the experimental autoimmune encephalitis (EAE) mouse model for MS, treatment with interferons raised the plasma levels of IL-10[17], suggesting that this anti-inflammatory cytokine is playing a role in the therapeutic mechanism.

The induction of plasma IL-10 levels appeared to be an interesting feature of these type I interferon injections, since this interleukin not only reduces inflammation but has also been described as one of the strongest anti-atherogenic cytokines[18]. It suggested that an existing therapy may be applied in the treatment of atherosclerosis.

While the role of other cytokines of the interleukin, TNF and type II interferon family was already well documented, with pro-inflammatory cytokines most often promoting atherogenesis and anti-inflammatory cytokines attenuating plaque growth[19], the few studies on the effect of type I interferons showed conflicting results. In the earliest published study, Wilson et al. show how treatment of cholesterol-fed rabbits with type I interferons resulted in a further unexplained reduction in lesion size accompanied by a decreased plaque lipid content, while plasma lipids were unaffected[20]. However, the interferons used in this experiment were isolated from cell cultures without detailed identification or assessment of the purity of the isolate. Therefore, other factors influencing atherogenesis may have been administered along with the interferons, although in a previous study, treatment with two different interferon-inducing agents caused a similar effect on the rabbit plaques[21]. In a second study, a low dose of IFNα was given to hyperlipidemic $LDLR^{-/-}$ mice for five weeks. Here, the treatment accelerated atherogenesis, which is explained by the higher plasma levels of both cholesterol and triglycerides in treated compared to control mice[22]. A third and final experiment used a more aggressive, hypercholesterolemia-independent atherosclerosis model, surgically ligating the carotid artery in $ApoE^{-/-}$ mice and locally perfusing angiotensin II (AngII) through a mini-pump. Here, IFNβ treatment attenuated atherogenesis as well as the AngII-induced but not the ligation-induced vascular remodeling. In their control $ApoE^{-/-}$ mice without AngII and on a normolipidemic diet, IFNβ treatment did not affect plaque formation[23].

In our experiments[24], daily injections with IFNβ, in doses comparable to treatment studies in the EAE model, promoted atherogenesis in two different murine models, despite raised plasma IL-10 levels and without influencing plasma lipids. The mechanism involved a CCL5-dependent enhanced recruitment of leukocytes to the vessel wall at atherosclerosis-prone sites and a resulting sub-endothelial macrophage accumulation, as shown in both *in vitro* and *in vivo* experiments. Moreover, deleting the *IFNAR1* gene specifically in lysozyme M-expressing cells and thereby inhibiting IFN signaling in myeloid cells resulted in smaller lesions, indicating that also endogenous IFN contributes to atherogenesis.

Whereas many cell types can produce type I interferons in response to an array of specific stimuli, the endogenous IFNs in the plaque are believed to be predominantly produced by plasmacytoid dendritic cells (pDC). This subset represents about one third of all dendritic cells and has, compared to myeloid dendritic cells (mDC), a low presence of the CD11c membrane marker for DC's, a high expression of IL-3 receptor CD123 and a less dendritic phenotype. They also express less extracellular but more intracellular Toll-like receptors and are therefore more specialized in the detection of viruses as well as nucleotides derived from dying cells. These viral- and autoantigens induce a massive production and secretion of type I interferons[25].

Since the presence of IFN-producing pDCs is demonstrated in human plaques[25] and *in vitro* treatment of human macrophages with IFNβ resulted in an upregulation of

chemokines and chemokine receptors[24], similar to mice, it should be investigated if our findings in murine models can be translated to human atherogenesis. These novel findings may be of importance in the search for new diagnostic and therapeutic approaches. For obvious reasons of susceptibility to viral or bacterial infections[26] however, it will not be possible to inhibit interferon production, interfere with its signaling or deplete pDCs as long as no methods are developed yet to target these therapies specifically to the atherosclerotic plaque. Also the ability to upregulate the chemokine CCL5 may be essential in the reaction to other pathologies and can therefore not be inhibited systemically. One has to keep in mind as well that human lesions differ significantly from the murine models, being far more complex and especially developing over several years instead of a few weeks[27]. It is therefore still uncertain if the contribution of cell recruitment to human atherogenesis is equally important as in murine plaques.

A more direct consequence of our results is the awareness that type I interferon treatment may not be as harmless as previously reckoned. Perhaps short-time treatment, as applied in hepatitis or tumor patients, might not affect a slowly progressing disease that much, but care has to be taken when treating for extended periods. Therefore, a thorough assessment of the cardiovascular risks in IFN-treated MS patients and a comparison with patients receiving other treatment options should be made as soon as possible.

Moreover, our findings may shed new light on the mechanisms that cause the 50-fold increased risk of developing atherosclerotic cardiovascular disease in patients suffering from systemic lupus erythematosus (SLE)[28]. This systemic autoimmune disease is characterized by elevated plasma levels of type I interferons[29], which is thought to play a central role in its pathogenesis[30] and is therefore targeted in experimental and clinical attempts to treat SLE[31]. Although the correlation between SLE and atherosclerosis in mice and patients was known before, it was until now explained by the combined contribution of different atherosclerosis-promoting factors, including traditional risk factors as well as a chronic inflammatory environment, elevated levels of auto-antibodies, endothelial dysfunction, decreased vascular repair and increased levels of oxidized lipids[28,32,33]. We, and simultaneously with us also other groups, now postulate that the high plasma levels of type I interferons may be an additional and important risk factor[24,34,35].

Similarly to SLE, other autoimmune inflammatory diseases including psoriasis, psoriatic arthritis and rheumatoid arthritis combine an essential role for type I interferons in their pathogenesis with an increased cardiovascular risk[36-40]. Also in these diseases, interferon signaling may contribute to this risk, in combination with traditional risk factors and other immune mediators like IL-1[41] and TNF[42].

A yet unpublished observation in our experiment in which ApoE$^{-/-}$ mice were treated with IFNβ may potentially shed a light on the importance of macrophage recruitment during the course of atherogenesis. In our experimental approach, we initiated the daily IFN injections simultaneously with the surgical positioning of the collars around the carotid arteries, implicating that the elevation of plasma IFNβ levels in these mice coincided with the initiation of carotid atherogenesis. At that moment however, these ApoE$^{-/-}$ mice were already fed a high-fat diet for more than three weeks and therefore, plaques had already started to form in other, more hyperlipidemia-dependent locations like the aortic root, the innominate artery and the aortic arch. While IFN injections promoted atherogenesis proximal of the collar, as shown in Chapter 2, plaques in the aortic arch and innominate artery were unaffected by this treatment **(Figure 6.2)**. Since the plaque size in these locations was not assessed in our other models, there is no certainty that atherogenesis in these locations is similarly dependent on CCL5-mediated leukocyte recruitment as plaques in the aortic root. A more plausible and instinctive explanation however would be that this effect of IFN treatment on plaque growth is most outspoken in newly-formed plaques. Since early lesions are rapidly expanding through the recruitment of new cells, they may be more affected by CCL5 upregulation than the larger, more advanced lesions, in which plaque stability parameters become more relevant. More evidence is needed to support this theory, for example by assessing the number of newly-recruited macrophages in plaques of different stages and sizes with the ER-MP58 marker that was described in Chapter 3. The outcome will be of high importance when designing future experiments and therapies that target the cell recruitment to the lesions.

It is therefore also possible that the results of the IFN treatment we described in Chapter 2 are highly dependent on the experimental setup we applied. In our models, daily IFN injections were initiated at the expected start of the high-fat diet-induced plaque formation (in the LDLR$^{-/-}$ model) or immediately after positioning the collars (in the ApoE$^{-/-}$ model) and therefore coincided with the earliest atherogenesis. In future experiments, it has to be verified if a similar atherosclerosis-promoting effect can still be observed when the treatment starts later in the disease progress. In the more advanced plaques, IFN treatment may affect the plaque stability, for example through TRAIL-mediated promotion of apoptosis[43] or inhibition of smooth muscle cell proliferation[44], rather than promote further plaque growth.

Other future experiments should focus on the effect of type I interferons in other cell types within the atherosclerotic lesion. Repeating the studies of high-fat diet-induced atherosclerosis in LDLR$^{-/-}$ mice that have a cell type-specific deletion of IFNAR1 in smooth muscle cells (smooth muscle actin promoter[45]) or endothelial cells (Tie-2 promoter[46]) may reveal additional mechanisms in which IFNs contribute to plaque growth or (in)stability.

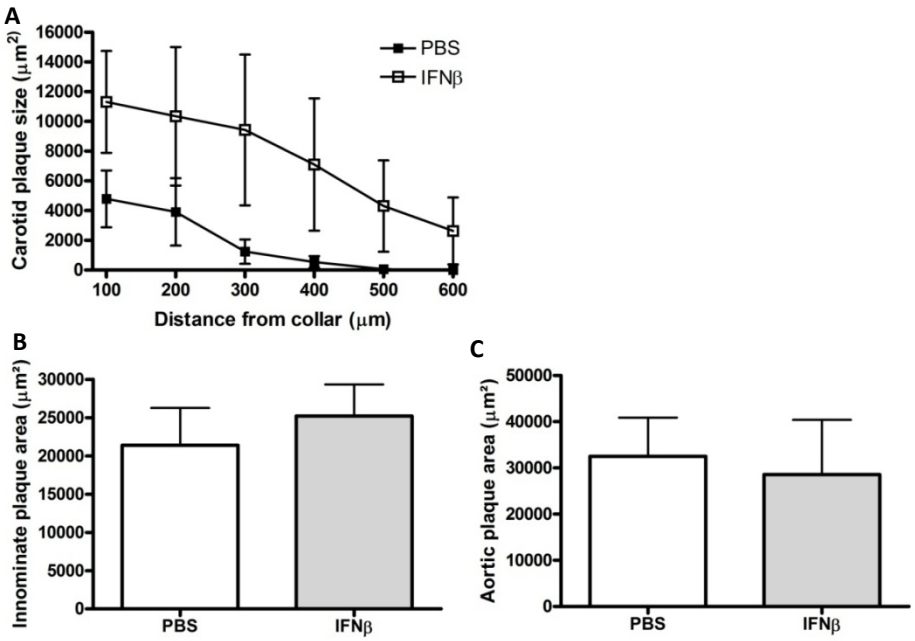

***Figure 6.2**: IFNβ treatment in ApoE$^{-/-}$ mice with a collar induced atherogenesis in the carotid artery (**A**) but did not affect plaque size in the innominate artery (**B**) nor in the transcersal aorta (**C**).*

Myeloid IκBα deficiency promotes atherogenesis

It has been shown extensively before that inflammation has a major effect on atherogenesis[4,47]. This is not only illustrated by the local presence of different inflammatory cell types[48] but also the proven influence of inflammatory cytokines on the development of the plaque[19]. The transcription factor NF-κB plays a key role in many of the cellular responses that drive inflammation, including the production of many of these cytokines[49]. This homo- or heterodimer is kept inactive through binding of the inhibitor of NF-κB (IκB). Phosphorylation of this inhibitor by the IκB kinase (IKK) complex and subsequently ubiquitination and degradation releases NF-κB and enables it to translocate to the nucleus, resulting in a specific gene expression pattern. This IKK complex can be activated through many different converging signaling pathways that respond to a wide range of stimuli, including microbial molecules, cytokines[50] and, notably in the atherosclerotic plaque, oxidized LDL particles[51] (Figure 6.1).

Because of its role in the initiation of inflammation and because atherosclerosis is an inflammatory disease, NF-κB activation was thought to contribute to the pathogenesis of this disease. In experiments targeting upstream signaling molecules that were previously published by our group and other groups however, it was shown that the role of NF-κB is far more complex and yet to be thoroughly investigated[52-54].

In **Chapter 3**, we aimed at further clarifying the effect of NF-κB activation in macrophages on murine atherogenesis by genetically and myeloid-specifically deleting its main inhibitor of the IκB family, IκBα. Contrary to the full IκBα$^{-/-}$ mice, these conditional LysMCre-IκBα$^{fl/fl}$ mice have no dysregulation of myelopoiesis[55]. Instinctively, they should result in a continuous NF-κB activation and a sustained NF-κB-dependent gene expression pattern in macrophages and neutrophils. However, both *in vitro* and *in vivo* experiments revealed disappointingly little increase in macrophage NF-κB translocation to the nucleus or upregulation of some typically NF-κB-dependent genes. Without clear experimental evidence for compensatory mechanisms, functional redundancy[56] or interference with other transcription factors[57] in this complicated and tightly regulated pathway, we still don't know yet why IκBα deletion cannot be used as a model for NF-κB hyper-activation. Knowing that the deletion of a directly binding inhibitor is still insufficient to deregulate this pathway, it can be questioned if it will even be possible to design genetic models of sustained NF-κB activation. Perhaps the closest useful approach would involve mice with a myeloid-specific deletion of the de-ubiquitinating enzyme A20[58], but one has to bear in mind that these mice also display a continuous IRF3 activation and thus produce elevated levels of the atherosclerosis-promoting type I interferons.

Remarkably, even without demonstrable effect on NF-κB activity, the chemokine CCL5 was upregulated in IκBα^{del} macrophages, promoting their adhesion to endothelial cells in a similar way as we described for IFNβ in Chapter 2. Although considered an NF-κB-dependent gene[59], we and others demonstrated the importance of IFNs in macrophage CCL5 expression[24,60]. This raises the question whether the observed upregulation is a consequence of the NF-κB or IFN signaling. As we have not detected an induced production of IFNs in the IκBα^{del} cells, future experiments may investigate whether these cells perhaps have an increased sensitivity to IFNs. In order to completely understand the effect of IκBα deletion on the gene expression pattern, a full micro-array should be performed on these macrophages, instead of the confined list of some typical NF-κB-dependent or atherosclerosis-influencing genes we assessed.

The introduction of ER-MP58 as a marker for newly-recruited macrophages in the plaque may offer new possibilities for further research. As mentioned above, quantification of the ER-MP58^{+} macrophages throughout the course of plaque development will bring valuable insights in the role of cell recruitment in different stages of the disease. It can also serve as a tool for assessing the effect of future therapeutic strategies and dietary changes in murine atherogenesis models.

A lentiviral vaccination targeting survivin potently reduces plaque size

In **Chapter 4**, a novel vaccination strategy for murine atherosclerosis was demonstrated. After confirming the presence of the mostly tumor-specific protein

survivin in newly-recruited macrophages of murine plaques, similarly to the human lesions[61], the evocation of a targeted immunity by injections with survivin-expressing lentiviral vectors successfully reduced diet-induced atherogenesis.

Despite the wide range of survivin-targeting therapeutic strategies that have been developed in tumor models and are already applied in the clinic[62], most of these approaches are aimed at a one-time elimination of survivin-positive cells and are therefore not applicable in the treatment of a chronic disease. Moreover, as discussed above, the recruitment of new macrophages to the lesion may contribute more to early pathogenesis and gradually reduce in importance when the plaques become more advanced. Survivin-targeting interventions would therefore be at their most effective against newly-forming plaques and thus should be prophylactic rather than therapeutic. This makes immune therapy the desired option for treating atherosclerosis, over the short-lived small-molecule antagonists, siRNA, anti-sense oligonucleotides and other survivin-targeting methods.

But although this vaccination strategy has been applied before in mice as well as in the clinic to combat survivin-expressing tumors with limited side effects and now appears to successfully target murine atherosclerosis as well, this therapy is not yet ready for application in atherosclerosis patients. First of all, one may question the ethics of vaccinating against a disease that affects a whole population but is, when well managed, invisible and non-terminal. On the other hand, vaccinating only the people that are proven to be at risk may come too late for optimal efficacy. Moreover, survivin-targeting vaccinations already were shown to be strongly varying in efficiency between different types of tumor cells, ranging from phase I trials that achieved a decrease in tumor markers in less than half of the treated patients[63] to patients that have temporary or even complete remission after vaccination[64]. Thence, the efficiency against plaque macrophages remains uncertain.

Also, the long-term effects of this vaccination should be assessed thoroughly first. Other than in cancer, where short-term and aggressive immunity against survivin should suffice to eliminate as many tumor cells as possible, usually in addition to other tumor-targeting therapies, inhibiting atherogenesis requires a life-long, latent immunity. Before this can be attempted, the role of survivin in non-tumoric, healthy cells should be investigated more exhaustively. However survivin is thought to be predominantly tumor cell specific and expressed only shortly and in low levels in healthy tissues[65,66], there may still be yet unknown cell type-specific and situation-specific requirements for survivin-expression[67], similar to the expression that was just recently discovered in plaque-infiltrating macrophages[61]. Certitude of the specificity is required before long-term immunity can be induced safely.

Before the above mentioned requirements can be met, a survivin-targeting immune therapy for atherosclerosis will not be ready for clinical application. Meanwhile, these results should rather be regarded as an illustration of the possibility to specifically

target cells in the atherosclerotic plaque, not only for vaccinations but potentially for plaque-specific delivery of other future therapeutics as well. Furthermore, immune histochemistry for survivin could be used in a similar way as described above for ER-MP58 to monitor macrophage recruitment to the plaques.

Future Perspectives

Other than a change in lifestyle and cholesterol-lowering drugs, no pharmaceuticals or therapies are yet available for intervening with the disease progression of atherosclerosis. Similarly to the difficulties in finding cancer treatments, the main problem may be that the cells we would wish to target are not differing enough from the healthy cells, while their functions we hope to block are still necessary in other, physiological processes. Therefore, it will be very important to focus further research on finding the properties that make the cells in the atherosclerotic plaque significantly different from healthy cells, enabling targeted therapies.

The abundance and the central role of macrophages in the plaque make this cell type the desired target for different therapeutic strategies[68]. From the very first phases of atherogenesis up until plaque rupture, macrophages drive the disease progression and influence the attraction and function of the surrounding cell types. Unlike the earliest proposed models of macrophage heterogeneity, with pro-inflammatory M1's and anti-inflammatory M2's, we now know that there is a wide range of macrophage phenotypes in between these two extremes and that each of these phenotypes is induced by their specific environment during differentiation, priming and activation[69]. Most likely, even the plaque macrophages are a collection of different phenotypes, depending on the cells' exposures to growth factors, cytokines, lipids etc. Therefore, macrophage markers related to the uptake and storage of lipids may be more attractive as a target than the classic M1 and M2 markers. Only a few genes are already described to be more or less specifically upregulated in (subsets of the) plaque macrophages, including the genes for the fatty acid binding protein aP2[70], the reverse cholesterol transporter ABCA1[71,72] and the nuclear receptor PPARγ[73,74]. Also the survivin gene is, as discussed earlier, upregulated in newly recruited plaque macrophages. Knowledge of these specific genes and pathways may allow therapeutic interventions but may also be useful for targeting vaccinations, like we and others demonstrated before[75,76], and as a target in other directed therapies. A recent publication from the oncology field for example illustrates the possibility of generating cytolytic viruses that specifically replicate in cells where a certain gene is expressed or pathway is induced[77].

As we explained in chapter 5, macrophage phenotype skewing *in vivo* through exposure to the right cytokines or other skewing stimuli may become a future therapeutic option for atherosclerosis[78]. A thorough knowledge of the phenotypes present in the lesion, their contribution to atherogenesis and the triggers needed to generate the desired

phenotype would be required, as well as a method to specifically affect the plaque macrophages while retaining the full functionality of macrophages in other tissues. This may be achieved by exploiting the above mentioned lesion-specific genes. For example, viral delivery of constructs that provide the aP2 or ABCA1 promoter regulating the IL-10 gene could trigger the expression of this anti-inflammatory cytokine specifically in the plaque macrophages and thereby induce an auto- and paracrine macrophage phenotype skewing and an anti-atherogenic effect[79]. While aP2 expression is independent of IL-10[80], ABCA1 expression is upregulated upon IL-10 signaling[81] and will therefore further amplify the cytokine's expression. Experimental evidence should clarify which of these options would be most successful, or if self-limiting mechanisms should rather be pursued.

In this thesis, the importance of leukocyte (and predominantly macrophage) recruitment to the plaque is an often recurring theme. Its modulation largely affects plaque growth, presumably most in newly forming lesions and to a lesser extent in more advanced stages of the disease. Reducing this recruitment by inhibiting chemokine secretion or blocking adhesion molecules slows down plaque growth[82-84] but it is important to remember that this recruitment is still essential in other functions of the macrophage, like infiltrating tissues upon the local detection of infectious agents. Therefore, general inhibition of recruitment is no option and more targeted solutions should be found[85].

Instead of interfering with the function of adhesion molecules and chemokines, it might be more useful to focus on the stimuli that induce their production instead, for example by skewing the plaque macrophages towards a phenotype that has a significantly reduced chemokine production. The endothelial cells' adhesion molecule production on the other hand could be reduced by targeting the source of the activation of these cells. As shown by Duewell et al[86], cholesterol crystals activate the NLRP3 inflammasome in plaque macrophages, leading to the activation and secretion of IL-1. Although the influence of this IL-1 on the surrounding cell types is still to be investigated, endothelial cells do express its receptor IL-1R and can, at least *in vitro*, be activated by IL-1[87]. Indeed, plaque size was reduced in mouse models with IL-1 deletion[88], IL-1R antagonist overexpression[89,90] or injections with this IL-1Ra[91]. The IL-1 production is rather specific for sterile inflammatory stimuli like cholesterol crystals, necrotic cells, asbestos and silica crystals but not for the microorganism-stimulated inflammatory response and subsequent cell recruitment[92]. It may therefore be an interesting target in future therapies aiming at the reduction of endothelial activation as a result of these stimuli while maintaining a proper response to the detection of microorganisms. Conveniently, IL-1 targeting therapies (e.g. anakinra) are already available in the clinic[93].

In conclusion, this thesis further illustrates the importance of macrophage recruitment in the pathogenesis of atherosclerosis. We show how an increase in the attraction of new macrophages to the lesion deteriorates the disease while specifically targeting these cells through vaccination reduces plaque size. These data may result in new therapeutic strategies for (early) atherogenesis. Also a thorough knowledge of the macrophages' inflammatory signaling and their intra-plaque heterogeneity will reveal new possibilities for the treatment of this disease, as described in this thesis.

References

1. Benoit, M., Desnues, B. & Mege, J.L. Macrophage polarization in bacterial infections. *J Immunol* **181**, 3733-3739 (2008).
2. Lawrence, T. Macrophages and NF-kappaB in cancer. *Curr Top Microbiol Immunol* **349**, 171-184 (2011).
3. Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175-184 (2007).
4. Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868-874 (2002).
5. Grundy, S.M.*, et al.* Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* **110**, 227-239 (2004).
6. Hlatky, M.A. & Bravata, D.M. Stents or surgery? New data on the comparative outcomes of percutaneous coronary intervention and coronary artery bypass graft surgery. *Circulation* **118**, 325-327 (2008).
7. Isaacs, A. & Lindenmann, J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* **147**, 258-267 (1957).
8. Borden, E.C.*, et al.* Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* **6**, 975-990 (2007).
9. Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H. & Schreiber, R.D. How cells respond to interferons. *Annu Rev Biochem* **67**, 227-264 (1998).
10. Katze, M.G., He, Y. & Gale, M., Jr. Viruses and interferon: a fight for supremacy. *Nat Rev Immunol* **2**, 675-687 (2002).
11. Samuel, C.E. Antiviral actions of interferons. *Clin Microbiol Rev* **14**, 778-809, table of contents (2001).
12. Walther, E.U. & Hohlfeld, R. Multiple sclerosis: side effects of interferon beta therapy and their management. *Neurology* **53**, 1622-1627 (1999).
13. Heathcote, J. & Main, J. Treatment of hepatitis C. *J Viral Hepat* **12**, 223-235 (2005).
14. Chawla-Sarkar, M.*, et al.* Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* **8**, 237-249 (2003).
15. Kiladjian, J.J., Mesa, R.A. & Hoffman, R. The renaissance of interferon therapy for the treatment of myeloid malignancies. *Blood* **117**, 4706-4715 (2011).
16. Paty, D.W. & Li, D.K. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology* **43**, 662-667 (1993).
17. Rudick, R.A.*, et al.* Interferon beta induces interleukin-10 expression: relevance to multiple sclerosis. *Ann Neurol* **40**, 618-627 (1996).
18. Mallat, Z.*, et al.* Protective role of interleukin-10 in atherosclerosis. *Circ Res* **85**, e17-24 (1999).
19. Tedgui, A. & Mallat, Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* **86**, 515-581 (2006).
20. Wilson, A.C., Schaub, R.G., Goldstein, R.C. & Kuo, P.T. Suppression of aortic atherosclerosis in cholesterol-fed rabbits by purified rabbit interferon. *Arteriosclerosis* **10**, 208-214 (1990).
21. Kuo, P.T., Wilson, A.C., Goldstein, R.C. & Schaub, R.G. Suppression of experimental atherosclerosis in rabbits by interferon-inducing agents. *J Am Coll Cardiol* **3**, 129-134 (1984).

22. Levy, Z.*, et al.* Low-dose interferon-alpha accelerates atherosclerosis in an LDL receptor-deficient mouse model. *Eur J Intern Med* **14**, 479-483 (2003).
23. Zhang, L.N.*, et al.* Interferon-beta attenuates angiotensin II-accelerated atherosclerosis and vascular remodeling in apolipoprotein E deficient mice. *Atherosclerosis* **197**, 204-211 (2008).
24. Goossens, P.*, et al.* Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. *Cell Metab* **12**, 142-153 (2010).
25. Niessner, A. & Weyand, C.M. Dendritic cells in atherosclerotic disease. *Clin Immunol* **134**, 25-32 (2010).
26. Stetson, D.B. & Medzhitov, R. Type I interferons in host defense. *Immunity* **25**, 373-381 (2006).
27. Zadelaar, S.*, et al.* Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol* **27**, 1706-1721 (2007).
28. Kahlenberg, J.M. & Kaplan, M.J. The interplay of inflammation and cardiovascular disease in systemic lupus erythematosus. *Arthritis Res Ther* **13**, 203 (2011).
29. Pascual, V., Banchereau, J. & Palucka, A.K. The central role of dendritic cells and interferon-alpha in SLE. *Curr Opin Rheumatol* **15**, 548-556 (2003).
30. Nacionales, D.C.*, et al.* Deficiency of the type I interferon receptor protects mice from experimental lupus. *Arthritis Rheum* **56**, 3770-3783 (2007).
31. Yao, Y.*, et al.* Neutralization of interferon-alpha/beta-inducible genes and downstream effect in a phase I trial of an anti-interferon-alpha monoclonal antibody in systemic lupus erythematosus. *Arthritis Rheum* **60**, 1785-1796 (2009).
32. McMahon, M. & Hahn, B.H. Atherosclerosis and systemic lupus erythematosus: mechanistic basis of the association. *Curr Opin Immunol* **19**, 633-639 (2007).
33. Ma, Z.*, et al.* Accelerated atherosclerosis in ApoE deficient lupus mouse models. *Clin Immunol* **127**, 168-175 (2008).
34. Li, J.*, et al.* Interferon-alpha priming promotes lipid uptake and macrophage-derived foam cell formation: A novel link between interferon-alpha and atherosclerosis in lupus. *Arthritis Rheum* (2010).
35. Lood, C.*, et al.* Platelet transcriptional profile and protein expression in patients with systemic lupus erythematosus: up-regulation of the type I interferon system is strongly associated with vascular disease. *Blood* **116**, 1951-1957 (2010).
36. Yao, Y.*, et al.* Type I interferon: potential therapeutic target for psoriasis? *PLoS One* **3**, e2737 (2008).
37. Mrowietz, U. & Reich, K. Psoriasis--new insights into pathogenesis and treatment. *Dtsch Arztebl Int* **106**, 11-18, quiz 19 (2009).
38. Ramonda, R.*, et al.* Atherosclerosis in psoriatic arthritis. *Autoimmun Rev* (2011).
39. Crow, M.K. Type I interferon in organ-targeted autoimmune and inflammatory diseases. *Arthritis Res Ther* **12 Suppl 1**, S5 (2010).
40. Sozzani, S., Bosisio, D., Scarsi, M. & Tincani, A. Type I interferons in systemic autoimmunity. *Autoimmunity* **43**, 196-203 (2010).
41. Ikonomidis, I.*, et al.* Inhibition of interleukin-1 by anakinra improves vascular and left ventricular function in patients with rheumatoid arthritis. *Circulation* **117**, 2662-2669 (2008).
42. Silva, L.C., Ortigosa, L.C. & Benard, G. Anti-TNF-alpha agents in the treatment of immune-mediated inflammatory diseases: mechanisms of action and pitfalls. *Immunotherapy* **2**, 817-833 (2010).
43. Niessner, A.*, et al.* Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. *Circulation* **114**, 2482-2489 (2006).

44. Schirmer, S.H*., et al.* Blocking interferon {beta} stimulates vascular smooth muscle cell proliferation and arteriogenesis. *J Biol Chem* **285**, 34677-34685 (2010).
45. Regan, C.P., Manabe, I. & Owens, G.K. Development of a smooth muscle-targeted cre recombinase mouse reveals novel insights regarding smooth muscle myosin heavy chain promoter regulation. *Circ Res* **87**, 363-369 (2000).
46. Forde, A., Constien, R., Grone, H.J., Hammerling, G. & Arnold, B. Temporal Cre-mediated recombination exclusively in endothelial cells using Tie2 regulatory elements. *Genesis* **33**, 191-197 (2002).
47. Hansson, G.K. Inflammatory mechanisms in atherosclerosis. *J Thromb Haemost* **7 Suppl 1**, 328-331 (2009).
48. Weber, C., Zernecke, A. & Libby, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* **8**, 802-815 (2008).
49. Hatada, E.N., Krappmann, D. & Scheidereit, C. NF-kappaB and the innate immune response. *Curr Opin Immunol* **12**, 52-58 (2000).
50. Verstrepen, L*., et al.* TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. *Cell Mol Life Sci* **65**, 2964-2978 (2008).
51. Stewart, C.R*., et al.* CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol* **11**, 155-161 (2010).
52. Kanters, E*., et al.* Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* **112**, 1176-1185 (2003).
53. Gareus, R*., et al.* Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. *Cell Metab* **8**, 372-383 (2008).
54. Wolfrum, S., Teupser, D., Tan, M., Chen, K.Y. & Breslow, J.L. The protective effect of A20 on atherosclerosis in apolipoprotein E-deficient mice is associated with reduced expression of NF-kappaB target genes. *Proc Natl Acad Sci U S A* **104**, 18601-18606 (2007).
55. Rupec, R.A*., et al.* Stroma-mediated dysregulation of myelopoiesis in mice lacking I kappa B alpha. *Immunity* **22**, 479-491 (2005).
56. Cheng, J.D., Ryseck, R.P., Attar, R.M., Dambach, D. & Bravo, R. Functional redundancy of the nuclear factor kappa B inhibitors I kappa B alpha and I kappa B beta. *J Exp Med* **188**, 1055-1062 (1998).
57. Gao, Z., He, Q., Peng, B., Chiao, P.J. & Ye, J. Regulation of nuclear translocation of HDAC3 by IkappaBalpha is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. *J Biol Chem* **281**, 4540-4547 (2006).
58. Matmati, M*., et al.* A20 (TNFAIP3) deficiency in myeloid cells triggers erosive polyarthritis resembling rheumatoid arthritis. *Nat Genet* **43**, 908-912 (2011).
59. Bowen, G.P*., et al.* Adenovirus vector-induced inflammation: capsid-dependent induction of the C-C chemokine RANTES requires NF-kappa B. *Hum Gene Ther* **13**, 367-379 (2002).
60. Genin, P., Algarte, M., Roof, P., Lin, R. & Hiscott, J. Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors. *J Immunol* **164**, 5352-5361 (2000).
61. Blanc-Brude, O.P*., et al.* IAP survivin regulates atherosclerotic macrophage survival. *Arterioscler Thromb Vasc Biol* **27**, 901-907 (2007).
62. Ryan, B.M., O'Donovan, N. & Duffy, M.J. Survivin: a new target for anti-cancer therapy. *Cancer Treat Rev* **35**, 553-562 (2009).
63. Tsuruma, T*., et al.* Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. *J Transl Med* **2**, 19 (2004).

64. Wobser, M.*, et al.* Complete remission of liver metastasis of pancreatic cancer under vaccination with a HLA-A2 restricted peptide derived from the universal tumor antigen survivin. *Cancer Immunol Immunother* **55**, 1294-1298 (2006).
65. Velculescu, V.E.*, et al.* Analysis of human transcriptomes. *Nat Genet* **23**, 387-388 (1999).
66. Altieri, D.C. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer* **3**, 46-54 (2003).
67. Fukuda, S. & Pelus, L.M. Survivin, a cancer target with an emerging role in normal adult tissues. *Mol Cancer Ther* **5**, 1087-1098 (2006).
68. Saha, P.*, et al.* The monocyte/macrophage as a therapeutic target in atherosclerosis. *Curr Opin Pharmacol* **9**, 109-118 (2009).
69. Gordon, S. & Martinez, F.O. Alternative activation of macrophages: mechanism and functions. *Immunity* **32**, 593-604 (2010).
70. Linton, M.F. & Fazio, S. Macrophages, inflammation, and atherosclerosis. *Int J Obes Relat Metab Disord* **27 Suppl 3**, S35-40 (2003).
71. Soumian, S., Albrecht, C., Davies, A.H. & Gibbs, R.G. ABCA1 and atherosclerosis. *Vasc Med* **10**, 109-119 (2005).
72. Schmitz, G. & Kaminski, W.E. ATP-binding cassette (ABC) transporters in atherosclerosis. *Curr Atheroscler Rep* **4**, 243-251 (2002).
73. Neve, B.P., Fruchart, J.C. & Staels, B. Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis. *Biochem Pharmacol* **60**, 1245-1250 (2000).
74. Hsueh, W.A. & Law, R.E. PPARgamma and atherosclerosis: effects on cell growth and movement. *Arterioscler Thromb Vasc Biol* **21**, 1891-1895 (2001).
75. Hauer, A.D.*, et al.* Vaccination against TIE2 reduces atherosclerosis. *Atherosclerosis* **204**, 365-371 (2009).
76. van Wanrooij, E.J.*, et al.* Vaccination against CD99 inhibits atherogenesis in low-density lipoprotein receptor-deficient mice. *Cardiovasc Res* **78**, 590-596 (2008).
77. Breitbach, C.J.*, et al.* Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* **477**, 99-102 (2011).
78. Stoger, J.L., Goossens, P. & de Winther, M.P. Macrophage heterogeneity: relevance and functional implications in atherosclerosis. *Curr Vasc Pharmacol* **8**, 233-248 (2010).
79. Han, X., Kitamoto, S., Wang, H. & Boisvert, W.A. Interleukin-10 overexpression in macrophages suppresses atherosclerosis in hyperlipidemic mice. *Faseb J* **24**, 2869-2880 (2010).
80. Shum, B.O.*, et al.* The adipocyte fatty acid-binding protein aP2 is required in allergic airway inflammation. *J Clin Invest* **116**, 2183-2192 (2006).
81. Rubic, T. & Lorenz, R.L. Downregulated CD36 and oxLDL uptake and stimulated ABCA1/G1 and cholesterol efflux as anti-atherosclerotic mechanisms of interleukin-10. *Cardiovasc Res* **69**, 527-535 (2006).
82. Braunersreuther, V.*, et al.* Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* **27**, 373-379 (2007).
83. Galkina, E. & Ley, K. Vascular adhesion molecules in atherosclerosis. *Arterioscler Thromb Vasc Biol* **27**, 2292-2301 (2007).
84. Bursill, C.A., Choudhury, R.P., Ali, Z., Greaves, D.R. & Channon, K.M. Broad-spectrum CC-chemokine blockade by gene transfer inhibits macrophage recruitment and atherosclerotic plaque formation in apolipoprotein E-knockout mice. *Circulation* **110**, 2460-2466 (2004).
85. Koenen, R.R.*, et al.* Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med* **15**, 97-103 (2009).
86. Duewell, P.*, et al.* NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* **464**, 1357-1361 (2010).

87. Thieme, T.R., Hefeneider, S.H., Wagner, C.R. & Burger, D.R. Recombinant murine and human IL 1 alpha bind to human endothelial cells with an equal affinity, but have an unequal ability to induce endothelial cell adherence of lymphocytes. *J Immunol* **139**, 1173-1178 (1987).
88. Kirii, H.*, et al.* Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* **23**, 656-660 (2003).
89. Devlin, C.M., Kuriakose, G., Hirsch, E. & Tabas, I. Genetic alterations of IL-1 receptor antagonist in mice affect plasma cholesterol level and foam cell lesion size. *Proc Natl Acad Sci U S A* **99**, 6280-6285 (2002).
90. Merhi-Soussi, F.*, et al.* Interleukin-1 plays a major role in vascular inflammation and atherosclerosis in male apolipoprotein E-knockout mice. *Cardiovasc Res* **66**, 583-593 (2005).
91. Elhage, R.*, et al.* Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice. *Circulation* **97**, 242-244 (1998).
92. Rock, K.L., Latz, E., Ontiveros, F. & Kono, H. The sterile inflammatory response. *Annu Rev Immunol* **28**, 321-342 (2010).
93. Dinarello, C.A. Blocking IL-1 in systemic inflammation. *J Exp Med* **201**, 1355-1359 (2005).

Supplemental Figures

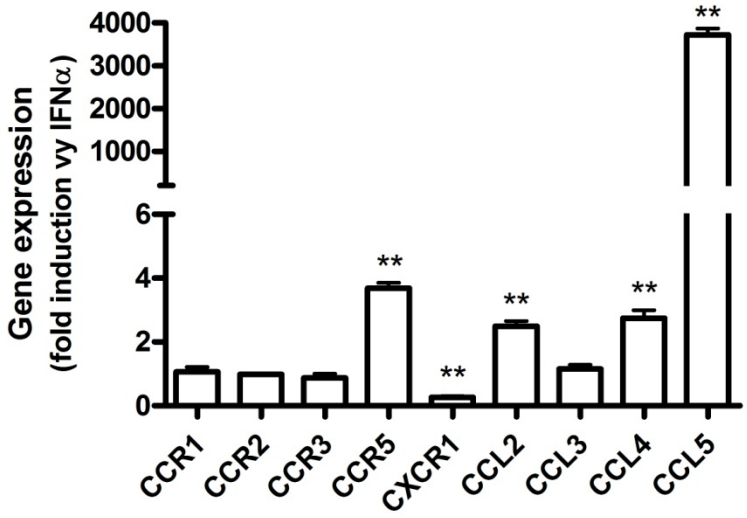

Figure S2.1: Expression of chemotactic factors after treatment with IFNα.

*Indicated is the fold-induction in bone marrow macrophages after 24h treatment with IFNα compared to untreated cells. Bars represent average of triplicate wells. (** p<0.01)*

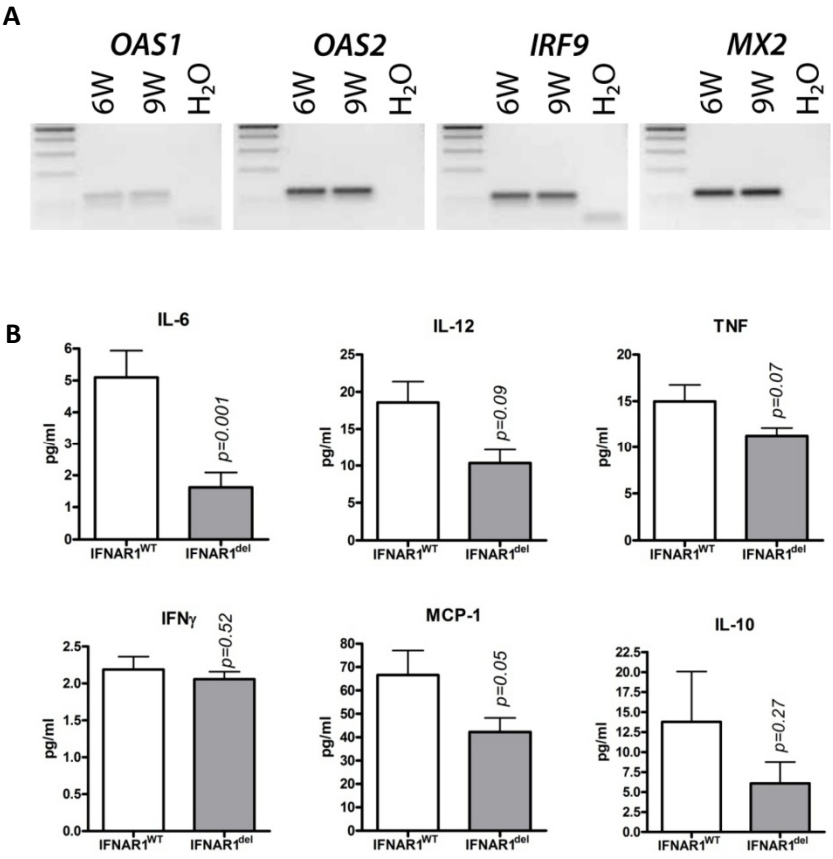

Figure S2.2: Expression of type I IFN signature genes in arches of atherosclerotic ldlr$^{-/-}$ mice and cytokine levels in* IFNAR1^{WT} *and* IFNAR1^{del} *mice after 8 weeks of high fat diet.

*(**A**) Expression of OAS1, OAS2, IRF9 and MX2 was detected in arches of ldlr$^{-/-}$ mice that had been fed a high fat diet for 6 (6W) or 9 (9W) weeks. (**B**) IL-6 levels are reduced in* IFNAR1^{del} *mice compared to* IFNAR1^{WT} *mice, while IL-12, TNF and MCP-1 show a non-significant trend towards a reduction. N=10 animals per group, P values are indicated.*

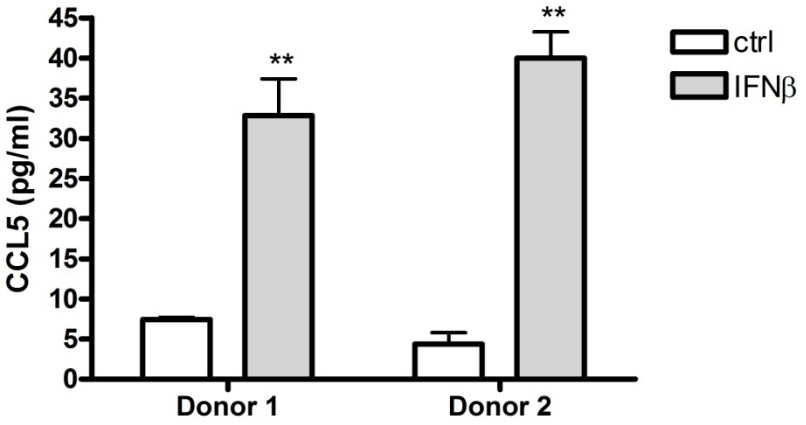

Figure S2.3: Secretion of CCL5 after treatment with IFNβ.

*Human primary macrophages were untreated (ctrl) or treated with IFNβ for 24h (IFNβ) and CCL5 was measured in the supernatant. Bars indicate average of triplicate wells. (** P<0.01)*

GeneSymbol	Fold Change Ruptured/Stable	Average *log*Intensity	Adjusted P-Value
Signalling pathway			
IFNAR1	1.27	9.62	4.03E-04
IFNAR2	1.45	9.00	1.99E-03
JAK1	0.99	9.05	9.29E-01
STAT1	1.92	10.42	4.91E-07
STAT2	1.17	11.57	4.12E-03
PTPN2	1.24	8.04	3.21E-06
TYK2	1.23	10.19	3.10E-04
Transcription products			
IFIT1	1.58	10.12	3.74E-04
IFIT3	1.72	9.38	3.08E-05
IFITM1	1.72	10.82	5.99E-04
MX1	1.51	12.19	1.50E-03
MX2	1.08	7.35	8.88E-05
OAS1	1.80	8.93	1.86E-06
OAS2	2.04	10.38	3.90E-06
PSMB8	1.36	8.83	1.69E-06
IRF9	1.14	11.20	1.24E-02
IFI35	1.47	9.88	9.85E-07
Chemokines			
CCR1	2.40	9.11	3.10E-08
CCR2	1.49	8.41	1.98E-06
CCR3	1.03	7.32	2.94E-03
CCR4	1.01	7.28	2.82E-01
CCR5*	5.58*		0.0040*
CX3CR1	1.00	8.02	9.78E-01
CCL2	1.88	11.85	1.60E-03
CCL3	2.33	11.06	2.67E-04
CCL4	Not detected on array		
CCL4L1	2.02	10.42	1.93E-04
CCL5	1.35	9.71	3.46E-02

Table ST2.1: Differential gene expression of Type I IFN signaling pathway and chemokines in human carotid plaques: Ruptured vs. Stable.

The Type I IFN signaling pathway is upregulated in unstable regions of human atherosclerotic lesions. Both genes involved in the Type I IFN signaling pathway as well as its target genes are mostly upregulated in ruptured human carotid plaques compared to stable regions. Fifty percent of the investigated chemokines are also upregulated in ruptured plaque regions.

** CCR5 was not detected on the array for unknown reasons, values presented are qPCR values relative to 18s rRNA of cDNA from 4 stable vs. 8 ruptured plaque lysates.*

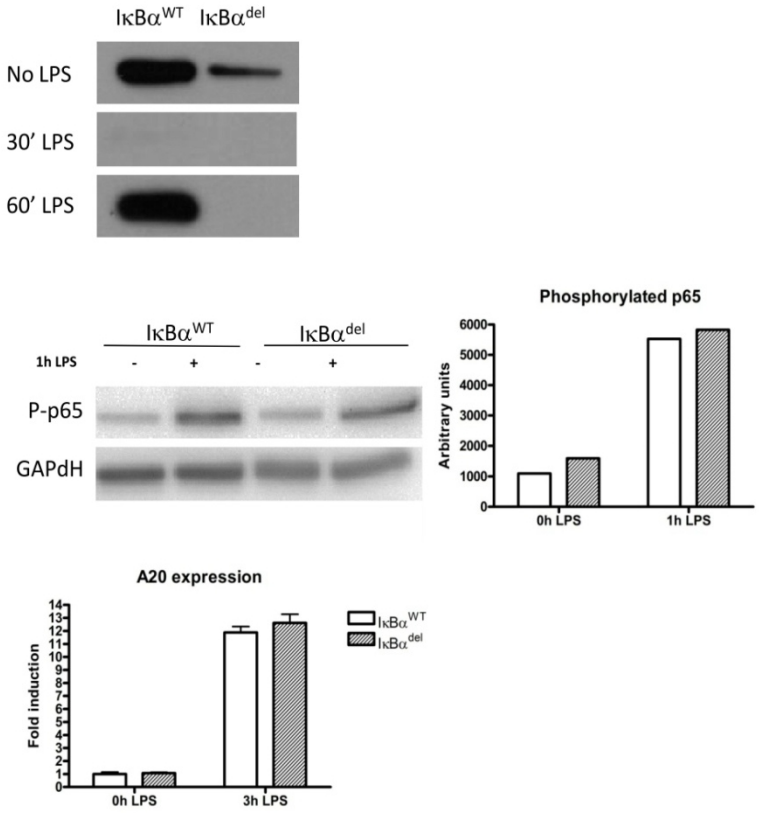

Figure S3.1: Deficiency of IκBα does not lead te increased NF-κB activity and subsequent gene expression.

*(**A**) Western blotting for IκBα on lysates of bone marrow derived IκBα^{WT} or IκBα^{del} macrophages confirmed a reduction in IκBα production, both before and after LPS activation. (**B-C**) A western blot for phosphorylated NF-κB subunit p65 showed that NF-κB is not continuously activated in IκBα^{del} macrophages, (**D**) resulting in a lack of differential expression patterns for NF-κB dependent genes.*

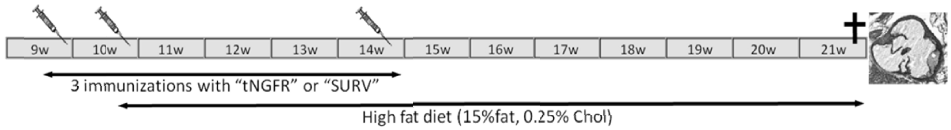

Figure S4.1: Schematic overview of the experimental setup used in the atherosclerosis vaccination study.

Each bar represents one week and indicates the age of the mice. Three immunizations with lentivirus were administered and the mice were fed a high-fat diet for 11 weeks. At the end of the experiment, mice were sacrificed and their atherosclerotic plaques were analyzed.

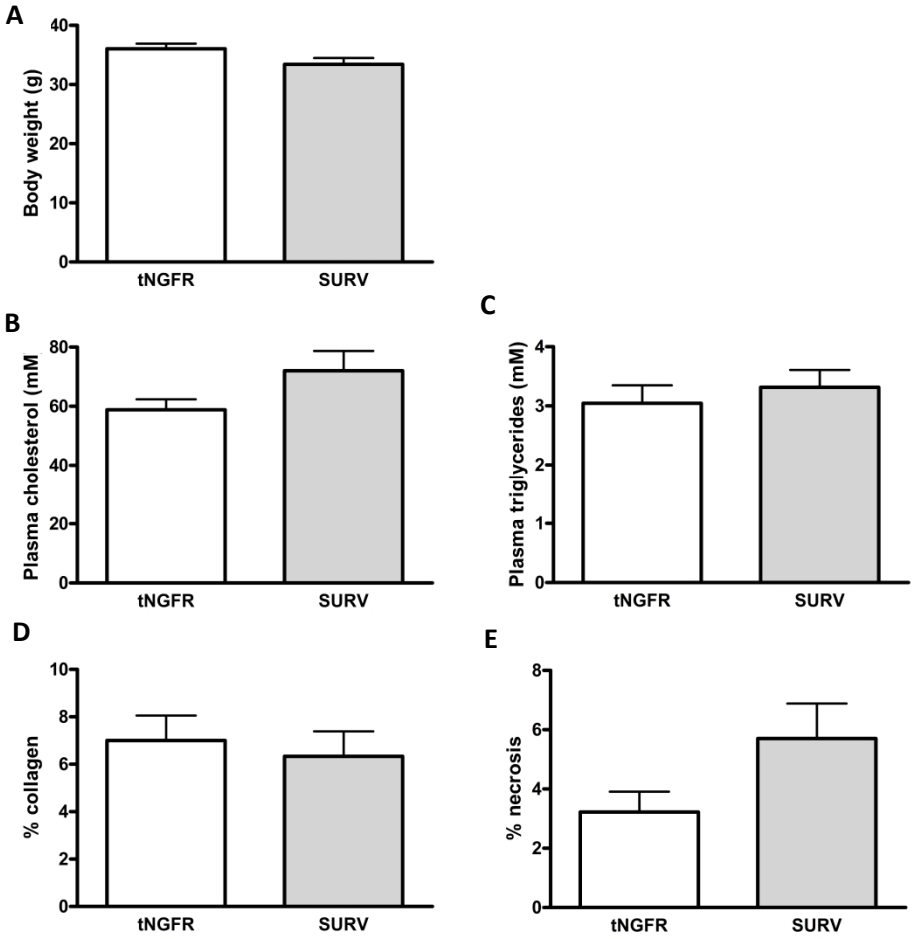

Figure S4.2: Body weight, plasma lipids and atherosclerosis parameters of vaccinated mice.

*(**A**) Body weight of the mice after 11 weeks of high-fat diet. (**B-C**) At the end of the experiment, blood was drawn from the mice and plasma cholesterol (B) and triglyceride (C) levels were measured enzymatically. None of these parameters showed significant differences. (**D-E**) Histochemical analysis of the lesions revealed no difference in plaque collagen content (D) and necrotic area (E).*

Summary

Cardiovascular events are the number one cause of death in western society and even gain quickly in importance since a disease-promoting lifestyle becomes more common. Obesity, high blood pressure, smoking and a lack of exercise are just some of the risk factors that contribute to atherosclerosis, the underlying pathogenesis of most of these cardiovascular diseases. While we have a fair idea of the cellular and molecular mechanisms that contribute to atherogenesis, treatment options are still mainly focused on reducing the risk factors.

The disease initiates when macrophages migrate to the vessel wall of an artery, where they take up modified lipids. This turns them into large, lipid-laden foam cells that cannot re-enter the blood stream but instead secrete factors that create a local inflammation, attracting even more macrophages and other immune cells. This accumulation of lipids and cells in the vessel wall is called an atherosclerotic plaque. In the chronic disease phase, the plaque will further grow until it ruptures and thereby forms a blood clot that can occlude downstream blood vessels. Depending on the location of the affected blood vessel, this results in acute manifestations such as ischemia, stroke or heart attack.

The macrophages are the most abundant cell type within the plaques and play a key role in all disease phases, from early atherogenesis to plaque rupture. This makes them potentially interesting targets for future therapies. Consequently, the experiments presented in this thesis mainly focus on the macrophages and their signaling pathways that are involved in the creation of an inflammatory environment as well as the recruitment of other cells to the plaque.

In **Chapter 2**, we investigated the effect of type I interferons (IFN) on the plaque macrophages. Although classically seen as antiviral mediators, it is becoming increasingly clear that these cytokines also play a major role in normal physiology and can act both as pro- or anti-inflammatory mediators in a variety of non-pathogen associated diseases. Using both treatment with IFNβ and myeloid specific knockouts for the type I interferon (IFN) receptor (IFNAR1) we have investigated the functional role of type I IFN in atherosclerosis in experimental models. We show that IFNβ enhances atherogenesis in two well established mouse models of atherosclerosis by upregulating chemokines and chemokine receptors that are necessary for atherosclerotic lesion development. In addition, we show that major targets for endogenous type I IFNs in atherosclerosis are myeloid cells, since mice with a myeloid specific deletion of IFNAR1 show reduced accumulation of myeloid cells in atherosclerotic lesions and thereby have reduced disease development. Moreover, we show that type I IFN signaling pathways are also activated in human atherosclerotic lesions and are associated with plaque rupture, making them potentially relevant for clinical outcome of disease.

It has been shown before that both the level of inflammation and the balance between pro- and anti-inflammatory mechanisms tremendously regulate the growth and composition of the atherosclerotic plaque. An important transcription factor involved in this regulation is NF-κB, which has indeed been described, by our group and others, to affect the process of atherogenesis. Its precise role(s) in the different cell types present in the plaque however remains unclear. In **Chapter 3**, the role of the NF-κB pathway in atherogenesis is further assessed by deletion of NF-κB's main inhibitor, IκBα, in the myeloid cells of a murine atherosclerosis model. It resulted in a two-fold increase in the size of the plaques. Further analysis suggests that myeloid IκBα deletion promotes atherogenesis by an enhanced recruitment of myeloid cells to plaques. This was supported by an immunohistochemical staining of the plaques with the ER-MP58 antibody, a novel method to quantify myeloid cell recruitment to the atherosclerotic plaque that we introduce in this chapter. This effect of myeloid IκBα deletion on murine atherogenesis is of high relevance in the further assessment of the role of inflammatory signaling pathways in the atherosclerotic plaque.

Chapter 4 features a new vaccination strategy in the treatment of experimental atherosclerosis, using a lentiviral vaccination approach to specifically target newly recruited macrophages in the atherosclerotic plaque. Evoking an immune reactivity against the antigen 'survivin' indeed resulted in a reduction of the plaque size and a local accumulation of cytotoxic T cells without causing adverse side effects.

Survivin is mainly known as a tumor-specific antigen since its expression in healthy cells is low but it is upregulated in virtually all tumors. Several survivin-based (gene) therapies targeting tumor cells have been developed in experimental models and some of them have already been applied in the clinic. Next to its tumor-specificity however, an article published a few years ago described that newly recruited macrophages in human atherosclerotic plaques also express survivin under the influence of local growth factors.

We confirm survivin expression in the newly recruited macrophages of murine plaques, target them using a vaccination strategy with a survivin-expressing lentivirus and demonstrate that this results in a significant reduction in lesion growth accompanied by an induction of recruitment of $CD3^+$ T cells and $CD8^+$ cytotoxic T cells to the plaque. This completely novel approach in experimental atherosclerosis may open up the potential of new therapies for this disease.

In **Chapter 5**, we describe the characteristics of different macrophage subsets and their specialized functions. While this wide range of phenotypes is already well described in other diseases with a key role for macrophages, like infection diseases and cancer, it most probably also affects atherogenesis. In this chapter we give an overview of the current relevant knowledge on macrophage heterogeneity and its putative roles in

disease progression, but also speculate on diagnostic and therapeutic possibilities that may benefit from it.

In conclusion, this thesis illustrates through different KO and treatment models the importance of macrophages and their inflammatory and chemotactic signaling pathways in the pathogenesis of atherosclerosis. Next to showing the disease promoting effect of enhanced recruitment of macrophages to the plaque, we also demonstrate that the specific targeting of these newly recruited cells may present a new strategy for plaque reducing therapies.

Samenvatting

Cerebrovasculaire accidenten zijn de voornaamste doodsoorzaak in de westerse samenleving en winnen nog steeds aan belang wegens het groeiend aantal mensen met een levensstijl die deze aandoeningen in de hand werkt. Obesitas, een verhoogde bloeddruk, roken en een gebrek aan lichaamsbeweging zijn slechts enkele van de risicofactoren die bijdragen tot atherosclerose, in de volksmond ook wel aderverkalking genoemd, de ziekte die de meeste van die accidenten veroorzaakt. En hoewel we al vrij goed op de hoogte zijn van de cellulaire en moleculaire mechanismen die verantwoordelijk zijn voor het ziekteverloop van atherosclerose, blijven de therapeutische opties voorlopig beperkt en wordt er vooral gefocust op het elimineren van de risicofactoren.

Atherogenese begint wanneer macrofagen vanuit het bloed naar de vaatwand van een slagader migreren, waar ze chemisch gemodificeerde lipiden opnemen. Hierdoor veranderen ze in zogenaamde schuimcellen. Deze grote, met vet gevulde cellen kunnen niet meer terug migreren naar de bloedbaan en zitten dus vast in de vaatwand. Van daar secreteren ze specifieke ontstekingseiwitten die nog meer macrofagen en andere immuuncellen rekruteren. Deze lokale ontstekingshaard en de opeenstapeling van cellen in de vaatwand wordt een atherosclerotische plaque genoemd. Gedurende de chronische fase van de ziekte zal deze plaque steeds verder groeien tot ze uiteindelijk openscheurt, waarbij zich een bloedklonter vormt die stroomafwaarts kleinere slagaders kan verstoppen. Afhankelijk van de slagader waarin de bloedstroom wordt afgesloten veroorzaakt dit acute problemen zoals lokaal zuurstoftekort, een beroerte of een hartaanval.

Een plaque bestaat voor een groot deel uit macrofagen en zij spelen dan ook een hoofdrol in elke fase van de ziekte, van de vroegste atherogenese tot het scheuren van de plaque. Dit maakt ze interessante doelwitten in de zoektocht naar nieuwe therapieën. De experimenten die in deze thesis worden beschreven focussen daarom voornamelijk op de rol van macrofaag signaaltransductie in het creëren van een inflammatoire omgeving en het aantrekken van nieuwe cellen naar de plaque.

In **Hoofdstuk 2** gaan we dieper in op het effect van type I interferonen (IFN) op plaquemacrofagen. Hoewel deze cytokines vooral zijn bestudeerd omwille van hun antivirale effecten, krijgen hun fysiologische functies steeds meer aandacht en is het duidelijk dat ze ook in andere, niet-infectieuze ziektes een rol spelen die zowel pro- als anti-inflammatoir kan zijn. Onze studie van deze type I interferonen in atherosclerose bestond zowel uit KO- als behandelingsexperimenten: in twee verschillende muismodellen voor atherosclerose konden we aantonen dat een dagelijkse behandeling met IFNβ de atherosclerotische plaquegroei stimuleert via een verhoogde productie van enkele chemokines en chemokinereceptoren die essentieel zijn in de

plaquevorming. Daarnaast beschrijven we hoe, binnen de plaque, myeloïde cellen het belangrijkste doelwit zijn van endogene interferonen, aangezien muizen waarin de receptor voor IFN (IFNAR) specifiek ontbreekt in de beenmerg afgeleide cellen minder accumulatie van cellen in de vaatwand vertonen en daardoor een vertraagd ziekteverloop hebben. Ten slotte bekijken we ook atherosclerotische plaques uit patiënten, waarin de IFN-signalering ook is geactiveerd en kan worden geassocieerd met het scheuren van de plaque. Dit wijst op een mogelijke klinische relevantie die verder onderzoek vereist.

Het is reeds vaak beschreven dat de groei en samenstelling van de atherosclerotische plaque zeer afhankelijk is van de mate van ontsteking alsook van de balans tussen pro- en anti-inflammatoire mechanismen. Bij de regulatie hiervan is een centrale rol weggelegd voor de transcriptiefactor NF-κB, waarvan inderdaad eerder al door zowel onze groep als andere groepen een invloed op het atherogeneseproces is beschreven. Desondanks blijft het precieze mechanisme in de verschillende celtypes van een plaque voorlopig onduidelijk. In **Hoofdstuk 3** wordt deze rol van NF-κB verder onderzocht door middel van een muismodel waarin IκBα, de belangrijkste inhibitor van NF-κB, ontbreekt in de beenmergafgeleide cellen. Het resultaat is een verdubbeling van de plaquegrootte in de muizen met IκBα-deletie. Verdere analyse van deze plaques suggereert dat dit kan worden verklaard door een verhoogde rekrutering van myeloïde cellen naar de plaques. Dit wordt bevestigd door immunohistochemische kleuringen van de plaques met het antilichaam ER-MP58, een in dit hoofdstuk geïntroduceerde methode voor het bepalen van de hoeveelheid nieuw-gerekruteerde macrofagen in een plaque. Dit effect van myeloïde IκBα-deletie kan van groot belang zijn in de verdere studies naar de rol van inflammatoire signalering in plaque-ontwikkeling.

In **Hoofdstuk 4** introduceren we vaccinatie met lentivirussen die een immuunreactie tegen het antigen ‘survivin’ induceren als een nieuwe strategie in de behandeling van experimentele atherosclerose. Door zo specifiek de nieuw-gerekruteerde macrofagen in de plaque te viseren konden we inderdaad de plaquegrootte reduceren zonder belangrijke neveneffecten te induceren. Survivin is vooral bekend als tumorspecifiek antigen aangezien het nauwelijks detecteerbaar is in gezonde cellen maar sterk tot expressie komt in zowat alle tumoren. Bij onderzoek in experimentele tumormodellen werd van deze eigenschap reeds gebruik gemaakt voor het ontwikkelen van verschillende survivin-gebaseerde (gen)therapieën, waarvan sommige intussen zelfs al succesvol in de kliniek worden toegepast. Een publicatie van enkele jaren geleden beschreef echter dat, naast deze tumorspecificiteit, nieuw-gerekruteerde macrofagen van humane plaques ook survivin tot expressie brengen onder invloed van lokale groeifactoren. Wij bevestigden de aanwezigheid van deze survivin-positieve, nieuw-gerekruteerde macrofagen in atherosclerotische plaques van de muis. Wanneer we

deze cellen gericht viseerden door middel van een vaccinatie met survivin-producerende lentivirussen, resulteerde dit in een significante vermindering van de plaquegroei en een verhoogde aanwezigheid van $CD3^+$ T-cellen en $CD8^+$ cytotoxische T-cellen in de plaques. Deze nieuwe behandelingsstrategie in experimentele atherosclerose zou het pad kunnen effenen voor nieuwe therapiemogelijkheden.

In **Hoofdstuk 5** beschrijven we de eigenschappen en specifieke functies van de verschillende macrofaagsubsets. Hoewel het bestaan van een brede waaier aan macrofaagfenotypes reeds uitvoerig is beschreven bij ziektes met een hoofdrol voor macrofagen, zoals infectieziektes en kanker, is dit concept hoogstwaarschijnlijk ook relevant voor de macrofagen in een atherosclerotische plaque. We geven daarom eerst een overzicht van de relevante kennis die over deze verschillende macrofagen bestaat, relateren dit aan de gekende macrofaagfuncties in een plaque en beschrijven tot slot enkele mogelijke diagnostische en therapeutische toepassingen.

Samenvattend illustreert deze thesis in verschillende KO- en behandelingsmodellen het belang van macrofagen en hun inflammatoire en chemotactische signalering in de pathogenese van atherosclerose. Behalve een sneller ziekteverloop ten gevolge van een verhoogde rekrutering van macrofagen naar de plaque, tonen we ook dat een specifieke targeting van deze nieuw-gerekruteerde cellen een nieuwe strategie voor atherosclerose-therapieën kan zijn.

List of abbreviations

ACE	Angiotensin-converting enzyme
acLDL	Acetylated LDL
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ApoE	Apolipoprotein E
Arg1	Arginase-1
ATM	Adipose tissue macrophage
Bcl	B-cell lymphoma-encoded protein
bFGF	Basic fibroblast growth factor
BMM	Bone marrow-derived macrophage
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
COX	Cyclooxygenase
Cre	Cre recombinase
CT	Computed tomography
CTL	Cytotoxic T cell
Ctrl	Control mice/cells
CVD	Cardiovascular disease
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDG	Fluorodeoxyglucose
Flox	Flanked by loxP
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony stimulating factor
HDL	high-density lipoprotein
HSP	Heat-shock protein
IAP	Inhibitor of apoptosis protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFNAR	Interferon-α/β receptor
IFNGR	Interferon-γ receptor
IκB	Inhibitor of NF-κB

IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
iNOS	Inducible nitric oxide synthase (=NOS2)
IP3	Inositol triphosphate
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
JAK	Janus kinase
KO	Knock-out
LDL	low-density lipoprotein
LDLR	LDL receptor
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LysM	Lysozyme M
MAP	Mitogen-activated protein
MAPK	MAP kinase
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
mLDL	Modified low-density lipoprotein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MR	Mannose receptor
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
Mx	Myxovirus resistance
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-κB
NK	Natural killer cell
NKT	Natural killer T cell
NLRP3	NOD-like receptor family, pyrin domain containing 3 (=NALP3)
NO	Nitric oxide
OAS	2'-5'-oligoadenylate synthetase
oxLDL	Oxidized LDL
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PET	Positron emission tomography
PKC	Protein kinase C

PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand 1
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SPECT	Single-photon emission computed tomography
SR-A	Class A scavenger receptor
STAT	Signal transducer and activator of transcription
SURV	Survivin
sVSG	Soluble variant-specific surface glycoprotein
TAM	Tumour associated macrophage
TF	Tissue factor
TGF-β	Transforming growth factor β
Th1/2	T helper 1 or 2 cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
TZD	Thiazolidinediones
UPR	Unfolded protein response
USPIO	Ultrasmall superparamagnetic particles of iron oxide
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen 4
VSG	Variant-specific surface glycoprotein

Dankwoord

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Heel veel dank ben ik natuurlijk verschuldigd aan mijn ouders! Al van zeer jonge leeftijd werd leergierigheid erg gestimuleerd. Zo kon ik namen van componisten opsommen voor ik zelfs nog maar deftig kon spreken, kon ik lezen (en zelfs al wat schrijven, ook dankzij meter natuurlijk) voor ik dat op school zou leren, ging ik in de bibliotheek enkel bij de boeken van een hogere leeftijdscategorie zoeken en was ik in een opstel zelden of nooit op een taalfout te betrappen. Toen ik in het zesde studiejaar de vraag kreeg wat ik in het secundair zou gaan studeren antwoordde ik dan ook met de vraag “Wat is het moeilijkste?”, en ook zes jaar later heb ik me niet neergelegd bij het studieadvies maar koppig hoger gemikt (bedankt dat jullie mij toen ook het vertrouwen gegeven hebben). Ongetwijfeld pluk ik nu nog steeds de vruchten van die onverzadigbare nieuwsgierigheid, en in tijden waarin alles wat al gekend is eenvoudig op te zoeken valt ga ik dan maar op zoek naar dingen die nog niet ontdekt zijn. Die zoektocht leidde mij ook steeds verder weg van huis: van het schooltje op ’t Keur naar het college in Dendermonde, van daar naar Brussel en dan via Maastricht nu naar Marseille. Gelukkig (voor ons moederke) is dit nog steeds te overbruggen met de auto, al zullen jullie mij nu ongetwijfeld nóg minder zien. Maar ooit kom ik wel weer wat dichter wonen hoor…

Zus, amuseer u met de Sisters maar vooral ook met Björn. En vergeet niet om regelmatig eens op bezoek te komen, kies u maar al eens een match van l’OM uit waar we samen naar toe kunnen!

Ook de rest van mijn familie is heel erg bedankt voor alle hulp en interesse! Sinds een paar jaar hoort daar ook mijn schoonfamilie bij, ik ben blij dat ik me zo welkom mag voelen bij jullie!

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Curriculum vitae

Pieter Goossens was born on September 11, 1981, in Dendermonde (Belgium). In 1999 he acquired his secondary school diploma in Latin – Sciences at the Heilige Maagdcollege (Dendermonde, Belgium). In September of the same year, he started to study Biomedical Sciences at the VUB (dutch-speaking Free University of Brussels) in Jette, Belgium, obtaining his Bachelor (candidate) diploma in 2001. In 2003, he got his Master (licentiate) degree cum laude after performing an internship of 10 months in the Laboratory for Molecular and Cellular Therapy at the Department of Immunology and Physiology of the VUB (Jette, Belgium). Under the supervision of Dr. Karine Breckpot, Lieven Straetman and Prof. Dr. Kris Thielemans he investigated the then newly-discovered protein survivin as a target in dendritic cell-based cancer immune therapy and as a potential tool for cell immortalization. In agreement with the university, this Master's degree was further complemented with a one-year custom made program, combining selected courses from the curriculum of the 2nd and 3rd year of the Bio-Engineer studies (specialization Cell and Gene Biotechnology) of the VUB in Etterbeek, Belgium. These courses included Microbiology, Virology, Parasitology, Immunology and Molecular Biotechnology.

In 2004, Pieter started working as a research fellow at the Laboratory of Cellular Microbiology of the Pasteur Institute Brussels (Belgium), under the supervision of Dr. Michael Kalai (Pasteur Institute Brussels) and Dr. Rudi Beyaert (Department for Molecular Biomedical Research, VIB Gent, Belgium). Supported by grants of Les Amis de l'Institut Pasteur and the Jean et Rose Hoguet Foundation, he assisted in organizing this newly-established lab and started investigating the interaction of the hepatitis G virus with the antiviral and ER stress pathways of its host cells. Less than two years later however, this project was discontinued prematurely due to a lack of funding.

In 2006, Pieter joined the Macrophage Group Maastricht of Dr. Menno de Winther in the Molecular Genetics department of the Maastricht University and the Cardiovascular Research Institute Maastricht (CARIM). His research on the role of macrophages and their inflammatory signaling in mouse models of atherosclerosis led to the findings described in this thesis. These experiments were supported by a Marie Curie Research Training Fellowship and the Netherlands Heart Foundation. Next to his own research, Pieter also mentored several students during their internships in the lab. His results were published in high-impact journals and presented on conferences and in labs in the Netherlands as well as the USA, Canada, Germany, Denmark, France and Greece.

Currently, Pieter is working in Marseille (France), in the Inflammation Biology Group of Dr. Toby Lawrence at the Centre d'Immunologie de Marseille-Luminy (CIML). Here, he is studying the phenotype of tumour-associated macrophages and the regulation of their inflammatory signaling pathways.

List of publications

Goossens P*, Stöger JL*, De Winther MPJ (*equally contributing)
Macrophage heterogeneity: relevance and functional implications in atherosclerosis
Current Vascular Pharmacology 2010, Mar; 8(2): 233-48 (Review)

Goossens P, Gijbels MJJ, Zernecke A, Eijgelaar W, Vergouwe MN, Van der Made I, Beckers L, Buurman WA, Daemen MJAP, Kalinke U, Weber C, Lutghens E, De Winther MPJ
Myeloid type I interferon signaling promotes atherosclerosis by accelerating macrophage recruitment to lesions
Cell Metabolism 2010, Aug; 12(2): 142-53

Lievens D, Zernecke A, Seijkens T, Soehnlein O, Beckers L, Munnix I, Wijnands E, **Goossens P**, Van Kruchten R, Thevissen L, Boon L, Flavell RA, Noelle RJ, Gerdes N, Biessen EA, Daemen MJAP, Heemskerk JWM, Weber C, Lutgens E
Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis
Blood 2010, Nov; 116(20): 4317-27

Goossens P, Vergouwe MN, Curfs D, Van Woezik JHG, Van der Made I, Rupec RA, Hofker M, Gijbels MJJ, De Winther MPJ
Myeloid IκBα deficiency induces macrophage accumulation in atherosclerotic lesions
PLoS ONE 2011, 6(7):e22327

Submitted publications

Goossens P, Van Puijvelde GH, Wolfs IMJ, Gijbels MJJ, Breckpot K, Straetman L, Heirman C, Donners MPC, Bot I, Thielemans K, Kuiper J, De Winther MPJ
A lentiviral vaccination strategy targeting the tumor antigen survivin reduces murine atherogenesis

Highlighted publications

Bird L
Macrophages: Interferons promote artery lesions
(Highlighting **Goossens** et al., Cell Met 2010)
Nature Reviews Immunology 2010 Sep; 10, 616-617

Published abstracts

Goossens P, Gijbels MJJ, Zernecke A, van der Made I, Beckers L, Buurman WA, Weber C, Kalinke U, Lutgens E, de Winther MPJ
Type I interferons promote atherosclerosis by affecting chemotaxis and cell death
Circulation 2008, 118: S 308

Goossens P, Vergouwe MN, Curfs D, Rupec RA, Gijbels MJJ, de Winther MPJ
Myeloid specific absence of IκBα aggravates atherosclerosis without altering plaque stability
European Journal of Immunology 2009 Sep; 39-S1

Fellowships & awards

Poster prize - Maastricht Medical Student Research Conference	(2003, Maastricht, NL)
Les Amis de l'Institut Pasteur Fellowship	(2004-2005, Brussels, BE)
Jean et Rose Hoguet Foundation Fellowship	(2005-2006, Brussels, BE)
Marie Curie Research Training Fellowship	(2006, European Commission)
Oral presentation award - Dutch Heart Foundation PhD meeting	(2007, Papendal, NL)
Poster prize - CARIM PhD student meeting	(2008, Maastricht, NL)
Young investigator award - Scandinavian Society for Atherosclerosis Research	(2008, Humlebæck, DK)
Invitation to present - ATVB Early Career reception	(2008, New Orleans, USA)
Oral presentation award - Dutch Atherosclerosis Society meeting	(2010, Ede, NL)

Oral presentations

Netherlands Heart Foundation PhD meeting	(2007, Papendal, NL)
11th Dutch Atherosclerosis Society meeting	(2008, Ermelo, NL)
Gesellschaft für Mikrozirkulation und Vaskuläre Biologie meeting	(2008, Aachen, DE)
American Heart Association Scientific Sessions	(2008, New Orleans, USA)
Cardiovascular Research Institute Maastricht meeting	(2009, Maastricht, NL)
Maastricht Genetics and Cell Biology plenary sessions	(2010, Maastricht, NL)
13th Dutch Atherosclerosis Society meeting	(2010, Ede, NL)
1st CARIM PhD workshop on publishing in high impact journals	(2010, Maastricht, NL)
La Jolla Institute for Allergy and Immunology seminar	(2010, San Diego, USA)
The Scripps Research Institute, Bruce Beutler lab seminar	(2010, San Diego, USA)
UC Berkeley Division of Immunology & Pathogenesis seminar	(2010, Berkeley, USA)
Centre d'Immunologie de Marseille-Luminy seminar	(2011, Marseille, FR)
Alexander Fleming Biomedical Sciences Research Center seminar	(2011, Athens, GR)

Poster presentations

7th Maastricht Medical Student Research Conference	(2003, Maastricht, NL)
Netherlands Heart Foundation PhD meeting	(2006, Papendal, NL)
10th Dutch Atherosclerosis Society meeting	(2007, Ermelo, NL)
Netherlands Heart Foundation PhD meeting	(2007, Papendal, NL)
Keystone Macrophage (D2) meeting	(2007, Copper Mountain, USA)
CARIM symposium	(2007, Maastricht, NL)
1st CARIM PhD student meeting	(2008, Maastricht, NL)
14th Scandinavian Society for Atherosclerosis Research meeting	(2008, Humlebæck, DK)
Netherlands Heart Foundation PhD meeting	(2008, Papendal, NL)
CARIM symposium	(2008, Maastricht, NL)
ATVB Early Career reception	(2008, New Orleans, USA)
Maastricht Genetics and Cell Biology plenary sessions	(2009, Maastricht, NL)
12th Dutch Atherosclerosis Society meeting	(2009, Ede, NL)
2nd European Congress of Immunology	(2009, Berlin, DE)
Keystone Atherosclerosis & Macrophage (J7 & J8) meeting	(2010, Banff, Canada)

Other conferences attended

Life, a Noble Story	(2004, Brussel, BE)
Maastricht Medical Student Research Conference	(2004, Maastricht, NL)
9th Dutch Atherosclerosis Society meeting	(2006, Ermelo, NL)
Immunology Research Maastricht session	(2006, Maastricht, NL)
Belgian Immunological Society meeting on macrophage biology	(2007, Gent, BE)
European Vascular Genomics Network workshop	(2008, Maastricht, NL)
CARIM PhD student weekend	(2008, Kerkrade, NL)
New Frontiers in Pattern Recognition Receptors symposium	(2009, Nijmegen, NL)
European Macrophage and Dendritic cell Society meeting	(2011, Brussel, BE)
FWO Meeting on Signaling in Inflammation and Immunity	(2011, Gent, BE)

Student's projects supervised

In vitro models for macrophage heterogeneity
Struijk R & Mattheij N, Bachelor Molecular Life Sciences (2007 – 2008)

Polarizing macrophages with helminth soluble egg antigens to combat atherosclerosis
Wolfs IMJ, Master Biomedical Sciences (2008 – 2009)

Unraveling the different signaling mechanisms in activated macrophage subsets
Hoeksema MA, Master Molecular Life Sciences (2009 – 2010)